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# **The Role of RecX in *Mycobacteria***

Sapna Lakhi Manglani

2009

A thesis submitted in partial fulfilment of the requirements of  
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for the degree of  
Master of Philosophy

Division of Mycobacterial Research,  
National Institute for Medical Research,  
The Ridgeway,  
Mill Hill,  
London.

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## ABSTRACT

Tuberculosis is a major global public health problem which causes millions of deaths every year. It is caused by the bacterium *Mycobacterium tuberculosis*, which survives and replicates within host macrophages, in which it has to overcome the effects of various DNA damaging agents in order to survive.

In bacteria, the RecA protein regulates DNA-damage repair. RecA also plays a central role in regulation of the SOS response and homologous recombination. In *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*, downstream of, overlapping and co-transcribed with *recA* is the *recX* gene. Using a *recA-recX* deletion mutant of *M. smegmatis*, it has been shown that the over expression of *recA* is toxic in the absence of *recX*, suggesting that RecX plays a regulatory role on RecA.

This project is aimed at investigating the hypothesis that RecX plays a regulatory role in RecA function in *Mycobacterium tuberculosis*. Various *in vivo* and *in vitro* studies were carried out to test this hypothesis and characterise the nature of RecA regulation by RecX.

To investigate their mode of interaction, the purified RecX protein was used in DNA mobility shift assays to look for interactions with the *M. smegmatis* and *M. tuberculosis* *recA* upstream regions. No interaction was observed, suggesting that RecX does not regulate *recA* expression at the transcriptional level. A positive interaction was found for RecA and RecX in a yeast two-hybrid study, demonstrating a post-translational mode of interaction and regulation.

The *in vitro* studies included LexA cleavage and ATP hydrolysis studies. When activated RecA is incubated with LexA, it results in the RecA-mediated autocatalytic cleavage of LexA. When RecX was added with RecA, a clear inhibition in LexA cleavage was observed. All reactions promoted by RecA *in vitro* are dependent on its binding to single-stranded DNA in the presence of ATP to produce an active nucleoprotein filament. It was found in ATPase assays, that RecX inhibits RecA ATP hydrolysis activity.

To investigate the function of RecX *in vivo*, an assay was carried out to determine whether *recX* promotes survival when expression of *recA* is induced by DNA damage. A *recA-recX* deletion mutant of *M. smegmatis* complemented with *M. smegmatis* *recA+recX* showed a higher percentage survival following DNA damage than when complemented with *recA* alone. Using the same *recA-recX* deletion strain of *M. smegmatis* in a homologous recombination assay, it was found that there is an increase in the frequency of double crossover events in *recA+recX* strains when compared to  $\Delta recX$  strains. Further *in vivo* studies demonstrated that *recX* enhances the recovery of RecA levels following pulse DNA damage.

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## ABBREVIATIONS LIST

|                   |  |
|-------------------|--|
| ADP               | Adenosine diphosphate                              |
| AP                | Apurinic/apyrimidinic                              |
| ATP               | Adenosine triphosphate                             |
| BCG               | Bacille Calmette-Guerin                            |
| bp                | Base pair  |
| BER               | Base Excision Repair                               |
| BSA               | Bovine serum albumin                               |
| CaCl <sub>2</sub> | Calcium Chloride                                   |
| CBP               | Calcium binding protein                            |
| cfu               | Colony forming unit                                |
| DCO               | Double crossover                                   |
| dH <sub>2</sub> O | Distilled water                                    |
| DMSO              | Dimethyl sulphoxide                                |
| DNA               | Deoxyribonucleic acid                              |
| dNTP              | Deoxynucleoside 5'-triphosphate                    |
| DTT               | Dithiothreitol                                     |
| EDTA              | Ethylenediaminetetraacetic acid                    |
| FOA               | 5-fluorouracil-6-carboxylic acid monohydrate       |
| g                 | Gram   |
| HEPES             | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |

|               |  |
|---------------|--|
| HIV           | Human Immunodeficiency Virus                 |
| Hyg           | Hygromycin                                   |
| HR            | Homologous recombination                     |
| IS            | Insertion sequences                          |
| IFN- $\gamma$ | Interferon gamma                             |
| IL-6          | Interleukin-6                                |
| IL-10         | Interleukin-10                               |
| IL-12         | Interleukin-12                               |
| IPTG          | Isopropyl thio- $\beta$ -D galactopyranoside |
| Kan           | Kanamycin                                    |
| KatG          | Catalase peroxidases                         |
| kb            | Kilobase pairs                               |
| kDa           | KiloDalton                                   |
| kV            | Kilovolts                                    |
| LAM           | Lipoarabinomannan                            |
| M             | Molar  |
| MDR TB        | Multi drug-resistant tuberculosis            |
| min           | Minute(s)                                    |
| mM            | Millimolar                                   |
| $\mu$ g       | Micrograms                                   |
| NER           | Nucleotide Excision Repair                   |
| ng            | Nanograms                                    |
| NHEJ          | Non-homologous end-joining                   |

|               |   |
|---------------|---|
| nM            | Nanomolar   |
| OD600         | Optical density reading at 600 nanometres                 |
| $\Omega$      | Ohms  |
| ONPG          | o-Nitrophenol $\beta$ -D-galactopyranoside                |
| ORF           | Open reading frame  |
| PCR           | Polymerase Chain Reaction                                 |
| PGLI          | Phenolicglycolipid I                                      |
| PNK           | Polynucleotide kinase                                     |
| rpm           | Revolutions per minute                                    |
| RNI           | Reactive Nitrogen Intermediates                           |
| ROI           | Reactive Oxygen Intermediates                             |
| RT-PCR        | Reverse transcription polymerase chain reaction           |
| s             | Seconds   |
| SCO           | Single crossover  |
| SDS-PAGE      | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SOD           | Superoxide dismutase                                      |
| TB            | Tuberculosis  |
| TGF- $\beta$  | transforming growth factor- $\beta$                       |
| TLS           | Translesion synthesis                                     |
| TNF- $\alpha$ | Tumour necrosis factor- $\alpha$                          |
| WT            | Wild-type strain  |
| XDR TB        | Extensively drug-resistant tuberculosis                   |



# CHAPTER 1

## INTRODUCTION

### 1.1 THE MYCOBACTERIA

The mycobacteria are members of the phylum Actinobacteria and the genus *Mycobacterium*, which comprises more than 71 known species. The mycobacteria are rod-shaped, aerobic, non-motile bacteria (Rastogi *et al.*, 2001). Most of the species of the mycobacteria are free-living in soil or water but the main ecological niche for some is the diseased tissue of hosts (David, 1973). An important feature of the mycobacteria is the relatively high guanine plus cytosine content of their genomic DNA, ranging from 62% to 70% (Tewfik and Bradley 1967).

Mycobacteria are classified as Gram-positive bacteria. Gram-positive organisms retain the stain because of the high amount of peptidoglycan in the cell wall and their cell walls typically lack the outer membrane found in Gram-negative bacteria. Mycobacteria are classified as Gram-positive bacteria even though they do not retain the crystal violet stain by Gram staining, but due to their lack of an outer cell membrane (Rastogi *et al.*, 2001).

Mycobacteria are surrounded by a complex cell envelope. This lipid-rich, hydrophobic envelope contains a high percentage of mycolic acids, which are branched fatty acids with long side chains. The nature of this cell wall results in these organisms being relatively resistant to chemical damage and to penetration by antibiotics. Such a cell wall confers obvious advantages to mycobacterial pathogens such as *Mycobacterium tuberculosis* (*M. tuberculosis*), which is spread as airborne droplets. The cell wall provides the bacteria with an acid-fast staining property, which is used to identify them. This means that once stained they resist decolorisation by acid or alcohol (Rastogi *et al.*, 2001).

The growth rates of the mycobacteria can range from generation times of 2 hours to 20 hours and this property has been used to classify the mycobacteria into two main groups, namely the fast growers and the slow growers. Classification according to Bergey's manual is that fast growers take up to one week to produce visible colonies on a plate while slow growers take much longer (Goodfellow *et al.*, 1986). The fast growers include non-pathogenic species such as *Mycobacterium smegmatis* (*M. smegmatis*), which is used as a model organism to study mycobacterial biology. The slow growers include species which cause disease in both humans and animals. This includes the causative organism of tuberculosis (TB), *M. tuberculosis*, and the causative organism of leprosy, *Mycobacterium leprae* (*M. leprae*) (Grange, 1996).

Evolutionarily *M. tuberculosis* is very closely related to *Mycobacterium bovis* (*M. bovis*) (Brosch *et al.*, 2002; Garnier *et al.*, 2003). There are phenotypic differences between

*M.tuberculosis* and *M.bovis* but by numerical taxonomy they are placed in a macro cluster, which is distinct from other slow growing mycobacteria (Baess, 1979). Genome sequencing has revealed that the *M.bovis* genome is 99.95% identical to that of *M.tuberculosis* and there are 11 deletions from the genome of *M. bovis* (Garnier *et al.*, 2003). The antigenic relatedness of these species also suggests that evolutionarily they are categorised as one species (Baess, 1979). The *Mycobacterium tuberculosis* complex presently consists of seven species and subspecies which include *Mycobacterium tuberculosis*, *Mycobacterium canettii*, *Mycobacterium africanum*, *Mycobacterium bovis*, *Mycobacterium microti*, *Mycobacterium pinnipedii*, and *Mycobacterium caprae*, characterized by more than 99.9% identity at the nucleotide level and an identical 16S rRNA sequence (Fleischmann., *et al.*, 2002).

## **1.2 MYCOBACTERIUM SMEGMATIS**

*M. smegmatis* was isolated by Trevisan, Lehmann and Neumann in 1899 (Gordon and Smith, 1953). The non-pathogenic bacillus rods are 3-5µm long and are curved and branching. The colonies tend to be rough, wrinkled or coarsely folded and creamy white in colour. This organism can grow from 25-45 °C. *M. smegmatis* provides a useful model to study various aspects of mycobacterial biology because, compared to other important pathogenic species such as *M. tuberculosis*, it has a much shorter generation time and work does not have to be carried out in biosafety level 3 containment facilities (Kahn and Scott, 1974).

### **1.3 MYCOBACTERIUM TUBERCULOSIS AND TUBERCULOSIS**

The *Mycobacterium tuberculosis* bacilli range in size from 0.3-0.6 x 1-4µm. These are straight or slightly curved rods, which grow in serpentine cord like masses, with a generation time of 14-15 hours and an optimum temperature of 37°C. The organism causes tuberculosis in man (Goodfellow *et al.*, 1986).

Tuberculosis kills more individuals than any other infectious disease in the world today, and the World Health Organisation has declared TB a global emergency. Factors such as the ease with which it is possible to travel, the increase in HIV and the occurrence of multi-drug resistant TB (MDR TB) have led to an increase in the incidence of tuberculosis (Chaisson and Slutkin, 1989). It is estimated that one third of the world's population is infected with TB (Kochi, 1991). It is important that the diagnosis of tuberculosis is done quickly because an undiagnosed and therefore untreated individual is easily able to infect several other individuals by casual contact. The common method to test for infection with *M. tuberculosis* is the tuberculin test. This involves testing for sensitivity to tuberculin, which is made of the crude or purified culture filtrates of *M. tuberculosis*. If infected, a hypersensitivity to tuberculin is seen. However, a reactive tuberculin skin test is also an indication of previous administration of the BCG vaccine against tuberculosis (Reddy *et al.*, 2002).

Another diagnostic method is the sputum test for which sputum is taken on three successive mornings as the number of organisms could be low, and the specimen is stained with Ziehl-Neelsen stain and viewed by microscopy. However the Ziehl-Neelsen

stain is positive in only 50% of cases, so even if no organisms are observed, further investigation is still required. Other methods include the use of fluorescent microscopy with auramine-rhodamine staining, growing *M. tuberculosis* on a selective medium, and chest X-rays of patients (Reddy *et al.*, 2002).

The recommended regimen for treating tuberculosis in an infected adult patient is with isoniazid, rifampin, pyrazinamide and ethambutol. The WHO has a recommended strategy for the detection and control of TB known as DOTS (Directly observed treatment, short-course). This involves a combination of political commitment, microscopy services, drug supplies, monitoring systems and the direct observation of treatment. According to this strategy, sputum smear positive cases detected by the microscopy services are then observed while taking their medication for six to eight months by health care workers. After two months of treatment, the sputum smear test is carried out and is checked again at the end of the treatment (Guy and Mallampali, 2008).

The treatment of tuberculosis usually takes several months before it is completed and a large number of individuals infected with the disease tend to live in countries with inadequate healthcare systems. Drug supplies may not be consistent and patients may not complete the dose due to lack of proper supervision. It has recently been found that some patients are resistant to all the currently administered drugs. These individuals are able to infect others and spread the resistant strains. MDR TB is TB resistant to at least the first line drugs isoniazid and rifampicin. When tuberculosis additionally develops resistance to any of the second-line anti-TB injectable drugs: Amikacin, Kanamycin or Capreomycin,

and to a fluoroquinolone then it is referred to as “extensively drug-resistant tuberculosis” (XDR-TB) (LoBue, 2009; Jassal and Bishai, 2009).

BCG (Bacille Calmette Guerin) is the vaccine used to prevent tuberculosis, first used in 1921. The vaccine was developed by Drs Calmette and Guerin by the growth of the bovine tubercle bacillus on glycerine-bile-potato medium, passaged 230 times until it was no longer able to cause disease in laboratory animals (Behr and Small, 1999).

Subsequently, progressive attenuating mutations may have been selected over time, or strains with deleted regions related to pathogenicity when BCG strains with fewer side effects were systematically chosen (Behr and Small, 1999; Nicod, 2007). Genetic studies have identified a region comprising 9 open reading frames, known as RD1, which is absent in all BCG stains and present in all *M. tuberculosis* strains. The genes within or controlled by RD1 are essential for *M. tuberculosis* virulence and the loss of RD1 is important in BCG attenuation (Lewis *et al.*, 2003; Pym *et al.*, 2002).

The efficiency of BCG as a vaccine against tuberculosis is variable in different populations, so the vaccine is used in some parts of the world and not others. There is a lack of efficacy in both low TB burden countries (USA) and high TB burden countries (India). A number of reasons have been proposed for the variation in efficiency. These include the genetic variation in the BCG strains used (Brosch *et al*, 2007), the difference in genetic make-up of different populations, exposure to environmental mycobacteria which results in a non-specific immune response against mycobacteria (masking) (Black *et al.*, 2002), immunity against mycobacteria which stops BCG from replicating and so

stops it from producing an immune response (blocking) (Brandt *et al.*, 2002) and differences in the virulence of the *M. tuberculosis* causing infection.

#### **1.4 THE *M. TUBERCULOSIS* GENOME**

The complete genome sequence of the virulent *M. tuberculosis* H37Rv strain was published in 1998 (Cole *et al.*, 1998). This was followed by the genome sequence of another *M. tuberculosis* strain CDC1551 (Fleischmann *et al.*, 2002) and others have followed.

*M. tuberculosis* H37Rv has a circular chromosome of 4,411,532 base pairs (bp) and consists of 3959 genes and 6 pseudo genes. Functions have been proposed for 52% of these genes (Camus *et al.*, 2002) and our knowledge about the other genes is growing constantly. Genes are characterised by various methods and techniques including bioinformatics, proteomics, functional genomics and comparative genomics.

Each gene in the sequence is represented by an individual Rv number and where a specific function is apparent, with a specific gene name. The genes have been classified into 11 different functional groups and among them 23% belong to the class known as conserved hypotheticals (Cole *et al.*, 1998), which show similarity to proteins of unknown function in other organisms.

The genome is abundant in genes involved in lipid metabolism and regulation (Tekaia *et al.*, 1999). Nearly 13% of the coding sequence is involved in cell wall synthesis and 6%

in lipid metabolism (Cole *et al.*, 1998). This is far greater than in *E. coli* and is reflective of the fact that the cell wall of mycobacteria is lipid rich (Domenech *et al.*, 2001).

About 7% of the coding capacity is represented by two large families of glycine and alanine-rich proteins, which may play a role in antigenic variation, facilitating bacterial replication in the macrophage (Ramakrishnan *et al.*, 2000). In addition, the genome contains 56 copies of insertion sequences (IS) belonging to nine different families (Gordon *et al.*, 1999). It has been suggested that the large number of insertion sequences are related to the bacterium's evolution (Sreevatsan *et al.*, 1997).

### **1.5 INFECTION WITH *M. TUBERCULOSIS***

*M. tuberculosis* is an intracellular pathogen that is able to survive and replicate in the alveolar macrophages. Upon infection by *M. tuberculosis* the host usually produces an immune response which is strong enough to prevent most people from developing the disease but not always enough to eliminate the infection (Flynn and Chan, 2001). The outcome represents the competition between the host immune system which is trying to protect the individual and the response of the bacterium against this system (Nathan and Shiloh, 2000)

Upon infection of the individual, most commonly by inhaling the organism into the lungs, the bacteria are phagocytosed by alveolar macrophages by a process involving macrophage mannose or complement receptors (Schlesinger, 1993; Noss *et al.*, 2001).



Generally phagocytosis leads to the production of phagosomes which fuse with lysosomes to form phagolysosomes.

The macrophage is a key cell in the destruction of invading bacteria. Micro organisms are degraded by intralysosomal acidic hydrolases, which function optimally at an acidic pH of 4.5-5.0 (Desjardins *et al.*, 1995). Upon entry into a macrophage, *M. tuberculosis* however is able to evade the hostile environment of phagolysosomes by the prevention of phagolysosomal fusion via interfering with normal phagosomal maturation, thus preventing lysis and enabling bacterial multiplication within the macrophages (Ferrari *et al.*, 1999; D'Arcy Hart *et al.*, 1987; Armstrong and Hart, 1975; Frehel *et al.*, 1986).

Interferon- $\gamma$  (IFN- $\gamma$ ) is the principal mediator of the process of macrophage activation. This leads to increasing the expression of various genes in the macrophage and greater presentation of antigens for recruiting T lymphocytes that participate in the destruction of bacteria; and promoting the production of nitric oxide (Ottenhoff *et al.*, 2005).

Activated macrophages generate reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI). ROI's are generated by oxidative bursts and RNI via NOS<sub>2</sub>-dependent cytotoxic pathway. RNI's such as nitric oxide and ROI's such as hydrogen peroxide have mycobacteriocidal effects (Yu *et al.*, 1999). Mycobacteria are able to evade the toxic response of ROI's and RNI's by various means such as the potent oxygen scavengers lipoarabinomannan (LAM), phenolic glycolipid I (PGLI), mycobacterial

sulfotides and also repair functions, mycobacterial catalase peroxidases (KatG) and superoxide dismutase (SOD) (Ehrt and Schnappinger, 2009).

The immune response involves cell migration and granuloma formation in the infected organ, controlled by adhesion molecules and chemokines. The granuloma consists of macrophages, giant cells, T cells, B cells and fibroblasts (Flynn and Chan, 2001). The state of the tubercle bacilli in the granuloma is unclear and could be dormant and non-replicating, replicating but killed off by the immune system, or metabolically altered with limited or infrequent replication cycles (Flynn and Chan, 2001).

Cell mediated immunity is important in the response to *M.tuberculosis* infection. It involves the activation of macrophages, natural killer cells and enabling them to destroy intracellular pathogens. It also involves activation of antigen-specific cytotoxic T-lymphocytes that are able to induce apoptosis in body cells displaying epitopes of foreign antigen on their surface, such as cells with intracellular bacteria. Cell mediated immunity also involves the release of various cytokines in response to antigens and is directed primarily at microbes that survive in phagocytes (Flynn and Chan, 2001).

Cytokines influence the function of other cells involved in adaptive immune responses and innate immune responses. During an infection by *M. tuberculosis*, various cytokines are induced. Cytokines are molecules produced and secreted by different immunocompetent cells after some stimulus, and are a central component in the immune

response against mycobacteria, participating in the regulatory processes, as well as in effector functions. These are mainly interleukin-12 (IL-12), interferon- $\gamma$  (IF- $\gamma$ ), interleukin-6 (IL-6), interleukin-10 (IL-10), tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and transforming growth factor- $\beta$  (TGF- $\beta$ ) (Lalvani *et al.*, 1998; Serbina and Flynn, 1999; Gong *et al.*, 1996; VanHeyningen *et al.*, 1997; Hirsch *et al.*, 1997).

CD4 cells are an important part of cell mediated immunity. They migrate to the lungs upon infection by *M. tuberculosis*, produce the cytokine IF- $\gamma$  and lyse infected macrophages (Feng *et al.*, 1999). CD4 is a co-receptor that assists the T cell receptor to activate its T cell following an interaction with an antigen presenting cell. CD4+ T cells and CD8+ T cells are both important in the response to a tuberculosis infection and are active in the tuberculous granuloma to contain the infection and prevent its reactivation (Feng *et al.*, 1999)

## 1.6 DNA REPAIR RESPONSES

*M. tuberculosis* cells reside within macrophages, in which oxidative stress produced through reactive oxygen metabolites such as reactive oxygen intermediates (ROI) and nitric oxide (NO) can lead to damage of lipids, proteins, and nucleic acids (Hassett and Cohen, 1989). The pathogen can develop nicks or lesions within the DNA, including single- and double-strand breaks, abasic, apurinic/apyrimidinic (AP) sites, which can then halt replication and cause the death of the pathogen (Davidsen *et al.*, 2007). The

organism must have adequate DNA repair mechanisms to overcome these effects of oxidative stress (Storz *et al.*, 1990; Buchmeier *et al.*, 1995).

DNA repair systems are crucial in maintaining the integrity of the genome (Drees, 2004b). DNA repair mechanisms also contribute to evolutionary changes and adaptive events resulting in antibiotic resistance. Therefore a study of these is significant to the development of therapeutic agents that can counteract *M. tuberculosis* infection (Dos Vultos *et al.*, 2009).

The DNA repair responses of *M. tuberculosis* are not as fully understood as in *E. coli* but information obtained from sequencing the pathogen's genome has identified a number of genes that are important through the recognition of homologues of genes involved in DNA repair in *E. coli* and related organisms (Mizrahi and Andersen, 1998). Genes required for nucleotide excision repair, base excision repair and recombination, as well as the SOS response and mutagenesis, have been identified in mycobacteria (Dos Vultos *et al.*, 2009). No homologs of genes involved in mismatch repair were identified, which is unlike other bacteria (Dos Vultos *et al.*, 2009).

The main types of bacterial DNA repair mechanisms that deal with damage caused by nicks or DNA lesions in mycobacteria are the Base Excision Repair (BER), Nucleotide Excision Repair (NER), Recombination repair, Non-homologous end-joining (NHEJ) and Translesion synthesis

(TLS) (Dos Vultos *et al.*, 2009). The descriptions of these mechanisms below are drawn on findings from work mainly with *E. coli*

### 1.6.1 Base Excision Repair

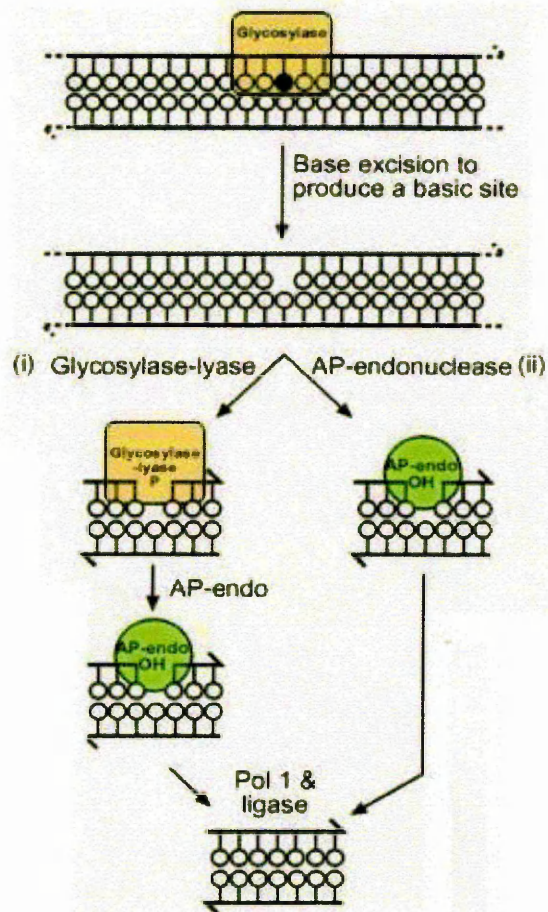
Oxidative DNA damage is primarily processed by the BER pathway (Seeberg *et al.*, 1995) (Figure 1). The BER mechanism involves a glycosylase protein which binds and removes a damaged base by nicking the N-glycosylic bond from the sugar phosphate backbone and a flipping mechanism, forming an AP site (Eisen and Hanawalt, 1999).

The phosphodiester backbone at the 5' end of the AP site is cleaved under the action of 5' AP endonucleases. This creates a free 3'-OH end for DNA polymerase repair synthesis (Mol *et al.*, 2000; Barzilay and Hickson, 1995). The sugar is removed and a repair patch of a single or a few nucleotides is synthesized. Deoxyribose phosphodiesterase removes the 5'deoxyribose-phosphate left by AP endonuclease (Seeberg *et al.*, 1995; Krokan *et al.*, 1997).

There are various DNA glycosylases such as the Ung protein which removes uracil, the mismatch specific uracil DNA glycosylase (MUG) protein which works on G-U and G-T mismatches (Eisen and Hanawalt, 1999), the MutY protein which cleaves the glycosylic bond of adenine from G:A, C:A base pairs (Nghiem *et al.*, 1988), the Nth protein which excises a variety of damaged pyrimidines, the Fpg protein, also known as MutM which

excises damaged purines (Cabrera *et al.*, 1988) and the AlkA glycosylase which excises many alkyl-base lesions (Eisen and Hanawalt, 1999). Examples of AP endonucleases include XthA and Nfo proteins (Eisen and Hanawalt, 1999).

**FIGURE 1: Base Excision Repair**



**An illustration of Base Excision Repair which is involved in DNA repair function.**

**This diagram is taken from Volkert and Landini (2001)**

### 1.6.2 Nucleotide Excision Repair

NER repairs bulky lesions which come about due to stress from exogenous sources. These lesions interfere with normal base-pairing and impair transcription and replication (Davidsen *et al.*, 2007, Hoeijmakers, 1993) (Figure 2).

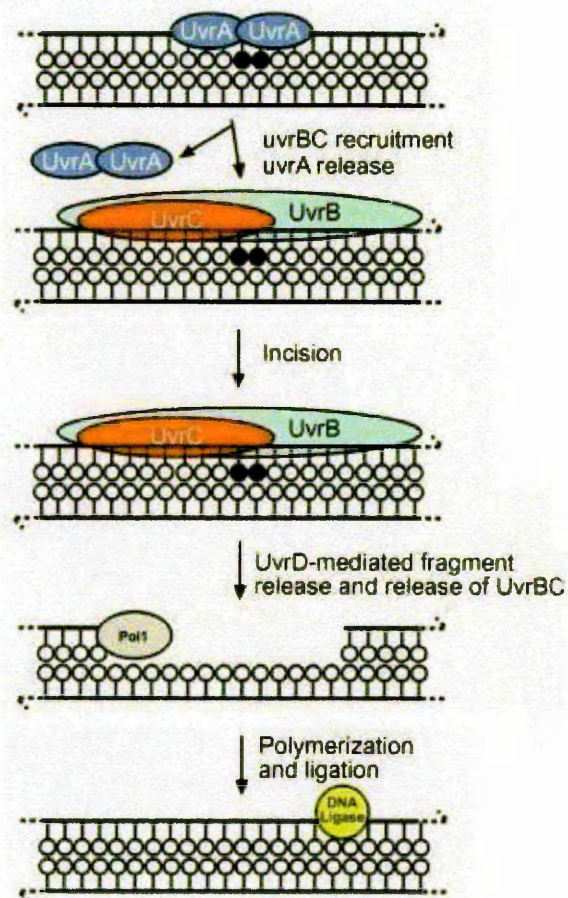
The mechanism involves the PolII, UvrA, B, C and D proteins. A complex consisting of a homodimer of UvrA protein plus UvrB protein detects disruptions in the DNA double helix, indicating the presence of a lesion (Darwin and Nathan, 2005). This complex binds DNA, UvrA recognizes the lesion and UvrB verifies that the lesion exists. The complex then separates the DNA strands at the lesion site. UvrA then dissociates and UvrB is left bound to the DNA. UvrC then binds to the UvrB-DNA complex, promoting the excision of a small fragment of the single stranded DNA on both the 5' and 3' sides surrounding the lesion. This produces dual incisions 12-13 nucleotides apart bracketing the lesion (Lin and Sancar, 1992; Moolenaar *et al.*, 2002, Eisen and Hanawalt, 1999). UvrD helicase then releases the fragment allowing DNA synthesis by PolII to fill the excised gap by resynthesis of a repair patch. This is followed by ligation of the contiguous strand at the end of the gap by DNA ligase (Volkert and Landini, 2001).

There is also a NER sub-pathway involving the Mfd protein which targets NER to the transcribed strand of actively transcribed genes. This mechanism is known as transcription coupled repair (Eisen and Hanawalt, 1999). It has been found that *uvrA*, *uvrB*, *uvrD* are part of the SOS response (described below) and induced by DNA damage in *E.coli*. The *uvrC* gene however is not a part of the SOS response and is not damage inducible (Van Houten *et al.*, 2002). Interestingly, the *uvrC* gene does exist in *M.*



*tuberculosis*, even though another *uvrC* homolog known as *Cho*, which is a part of the SOS response, also exists. It is not entirely clear why *UvrC* and *Cho* co-exist (Van Houten *et al.*, 2002).

**FIGURE 2: Nucleotide Excision Repair**



**An illustration of Nucleotide Excision Repair which is involved in DNA repair function.**

**This diagram is taken from Volkert and Landini (2001)**

### 1.6.3 Recombination repair

Double-stranded breaks caused by DNA damage are primarily repaired by recombinational repair by the RecABCD pathway (Kuzminov and Stahl 1999) (Figure 3). The RecA protein is a key component of this pathway (West *et al.*, 1981; Webb *et al.*, 1997, Kuzminov and Stahl, 1999). It is also necessary for induction of the SOS response, up-regulating the expression of the more than 40 genes in *E. coli* (Drees *et al.*, 2004b) (described in further detail below).

The RecA enzyme is a helical hexamer, which is 120 Å wide and consists of a central diameter of 25 Å. The central diameter consists of three domains, a major central domain, a smaller amino terminal, and a carboxy domain. RecA forms a RecA–ssDNA–ATP nucleoprotein filament and the central domain is involved in ATP, ssDNA, and duplex DNA binding, with the DNA binding regions being highly conserved (Mishra *et al.*, 2003) (Figure 4).

RecA has the ability to homologously pair, via strand exchange, a damaged duplex with the intact sister duplex. The homologous recombination process *in vitro* is the three-strand exchange reaction between circular single and linear double-stranded DNA which involves three stages. The first stage is the presynaptic polymerization of RecA protein on single-stranded DNA. This is followed by synapsis, which is the homologous

alignment of nucleoprotein filament with linear double-stranded DNA; and finally the unidirectional strand exchange stage (Venkatesh *et al.*, 2002).

When there is a lesion, upon DNA replication, the DNA replication apparatus stalls, resulting in single stranded DNA (Webb *et al.*, 1997). If it is a double stranded break the RecBCD complex unwinds and degrades the DNA in a 3' to 5' action simultaneously from the site of the break till a Chi site is reached, which then is able to stall the complex. RecBCD activity is modified such that the 5' single-stranded DNA is preferentially degraded, yielding a 3' single-stranded tail. Also, RecBCD plays an active role in loading RecA onto the Chi-terminated 3' single-stranded DNA produced (Andersen *et al.*, 1999).

In general, ssDNA arising in the cell is coated with ssDNA binding protein (SSB). This leads to the removal of secondary structures in the DNA which could impede the action of RecA (Kowaczykowski and Krupp, 1987; Webb *et al.*, 1997). However, this process also inhibits RecA from undergoing the nucleation event that allows RecA filaments to coat DNA (Shan and Cox, 1997, Lusetti *et al.*, 2004). RecF, RecO and RecR are involved in the loading of the RecA filament on SSB-coated single-stranded DNA (Drees *et al.*, 2004b). These proteins are able to bind the SSB-ssDNA complex, thereby modifying the complex structure and allowing the binding of RecA (Umezumi and Kolodner, 1994).

RecA functions as a nucleoprotein filament, with filaments assembling on single-stranded DNA (ssDNA) and under some conditions on duplex DNA (dsDNA). These filaments assemble and disassemble in the 5' to 3' direction on ssDNA. The protomers are added to one end and subtracted from the other end (Drees *et al.*, 2004b). The rate of extension of RecA filaments during assembly is faster than the end-dependent disassembly, thereby leading to the formation of long filaments (Lusetti *et al.*, 2004) (Figure 5).

The extension of RecA in a 5' to 3' direction then displaces SSB in an ATP dependent manner (Kowalczykowski and Krupp, 1987; Shan and Cox, 1997). RecA acts as a DNA-dependent ATPase (Drees *et al.*, 2004b; Lusetti *et al.*, 2004) in the form of this RecA-ssDNA-ATP nucleoprotein filament (Stohl *et al.*, 2003).

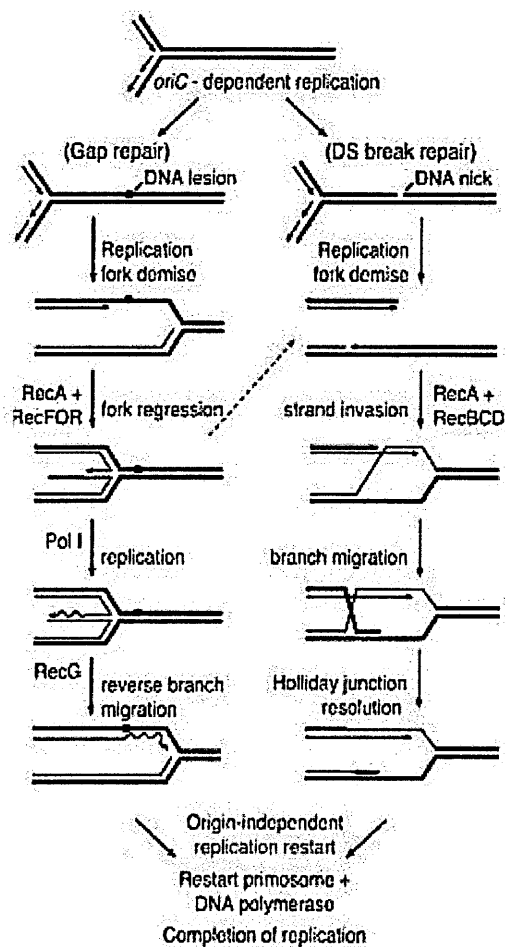
RecA is also auto-regulated by the RecA C-terminus, which is able to modulate the displacement of SSB by RecA (Eggler *et al.*, 2003) and is able to block the direct binding of RecA to duplex DNA, guiding RecA filament nucleation to single strand gaps (Benedict and Kowalczykowski, 1988; Lusetti *et al.*, 2004; Tateishi *et al.*, 1992; Drees, 2004b). RecF and RecO control the binding and filamentation of RecA, acting as a complex to stop extension beyond the single-stranded region (Webb *et al.*, 1997).

RecA bound to the DNA is able to align this DNA to a homologous sequence in double stranded DNA leading to the occurrence of strand exchange and the formation of a Holliday junction (Lindsley and Cox, 1990; Webb *et al.*, 1997).

Branch migration is then facilitated by RuvA and RuvB acting together as a complex, or by RecG (Shiba *et al.*, 1991; West *et al.*, 1981), allowing strand exchange to occur over long gaps in DNA (Iype *et al.*, 1994).

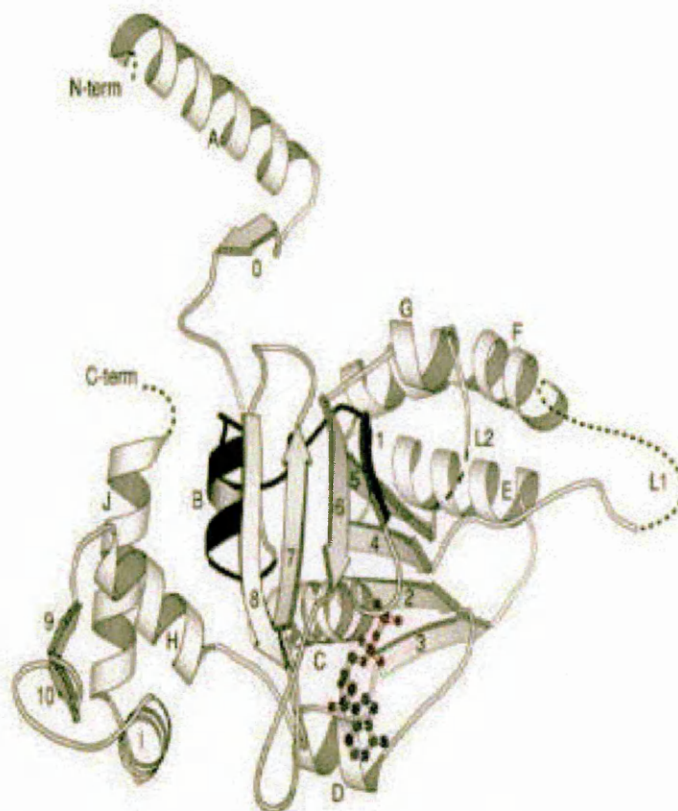
Following the completion of the extension of the new DNA strand, the Holliday junction is resolved by RuvC, which nicks strands of like polarity (West, *et al.*, 1981). The nick is then repaired by DNA ligase (West, *et al.*, 1981).

**FIGURE 3: Recombination Repair**



**A summary of the mechanism for recombination repair following DNA damage which stalls replication forks.**  
**Taken from Cox, 2001**

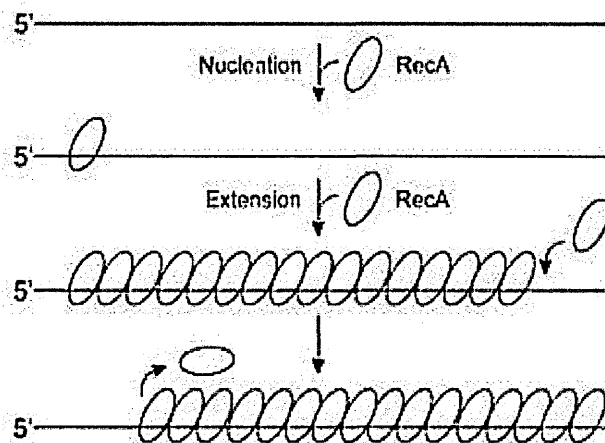
**FIGURE 4: Structure of the RecA Protein Monomer**



The structure of the RecA protein from *E. coli*. The structure includes a major domain and two smaller subdomains at the N and C termini.  
Taken from Story et al., 1992



**FIGURE 5: The assembly and disassembly of RecA on single stranded DNA**



The assembly of RecA protein on single stranded DNA involves nucleation, followed by extension in the 5' to 3' direction. Disassembly also occurs in the 5' to 3' direction, proceeding from the end opposite to that where extension occurs.

Taken from Cox, 2005

#### 1.6.4 Non-homologous end-joining

Double strand breaks can be caused by agents such as reactive oxygen species and are a threat to genomic DNA integrity and cell survival. The non-homologous end-joining (NHEJ) pathway is a double strand break repair pathway which requires the Ku protein and a multifunctional DNA ligase, LigD. These form a two-component repair complex at the termini of double strand breaks and are involved in the processes of break recognition, end processing and ligation for double strand break repair (Pitcher *et al.*, 2005). The NHEJ repair pathway requires minimal base pairing at the break junction. DNA ends are brought together and ligated directly in a relatively error-prone mechanism. The genetic alterations produced can be the loss or addition of several nucleotides to chromosome translocations (Krejci *et al.*, 2003).

The Ku protein is a homodimer that binds to the termini of double strand breaks (Weller *et al.*, 2002). In *B. subtilis* the Ku-like protein is encoded by *ykoV*. However in *Mycobacterium tuberculosis* the *ku* gene is genetically linked in an operon with another gene that encodes a putative ATP-dependent DNA ligase, which function together and are divergently transcribed: Mt-Ku (*rv0937c*) and ligase Mt-Lig (*rv0938*) (Aravind and Koonin, 2001; Weller and Doherty, 2001).

Following DNA damage, the Mt-Ku homodimer locates the double strand break (Krejci *et al.*, 2003) and binds to the broken DNA ends. Mt-Ku then recruits the multifunctional DNA repair ligase Mt-Lig to the double strand break (Dudasova *et al.*, 2004). The broken ends are then aligned by the complex followed by ligation of the break. When the DNA ends are non-complementary and/or are damaged, Mt-Lig carries out DNA end-

processing, gap filling and nucleolytic activities to generate ligatable DNA termini prior to ligation (Nick McElhinny *et al.*, 2000)

### 1.6.5 Translesion Synthesis

Translesion synthesis (TLS) is a mechanism to overcome replication blockage by DNA damage, by allowing DNA replication machinery to replicate past lesions persisting at the replication fork during DNA synthesis such as AP sites or thymine dimers. It involves using translesion polymerases to facilitate the insertion of bases opposite damaged nucleotides. These polymerases allow replication past blocking lesions but they lack 3'-5' exonuclease activity and often have low fidelity so this is carried out at the cost of mutations. This however may still be preferable to resorting to more drastic mechanisms of DNA repair, which may cause gross chromosomal aberrations or cell death (Napolitano *et al.*, 2000, Davidsen *et al.*, 2007; Beuning *et al.*, 2006, Indiani *et al.*, 2009).

TLS is mediated by three DNA polymerases in *E. coli*: Pol II (Bonner *et al.*, 1988), DinB (Wagner *et al.*, 1999), and UmuDC' (Wang, 2001) and several regulatory factors, including those encoded by the *umuD* gene that influence the activity of UmuC. The *umuC* and *umuD* genes are co-transcribed in *E. coli*. Both *dinB* and *umuDC* are regulated transcriptionally by the SOS regulatory network (Explained below) (Napolitano *et al.*, 1997).

Under conditions of DNA damage, DinB is the most abundant DNA polymerase in *E. coli* (Sutton *et al.*, 2001). However not a lot is known about its biochemical regulation. UmuD<sub>2</sub> is up-regulated 10-fold immediately after damage, after which it undergoes an SOS-mediated posttranslational modification involving the removal of the N-terminal 24 amino acids of each UmuD. This happens when it is subject to facilitated autocleavage upon interaction with RecA-ssDNA nucleoprotein filaments to yield UmuD'<sub>2</sub> (Burckhardt *et al.*, 1988; Nohmi *et al.*, 1988). The converted UmuD'<sub>2</sub> activates UmuC polymerase function (Reuven *et al.*, 1999). RecA-ssDNA filaments are then required for UmuD'<sub>2</sub>C-catalyzed translesion synthesis (Schlacher *et al.*, 2006). Following this, the RecF, RecO, and RecR proteins then function to alleviate the inhibition of UmuD'<sub>2</sub>C mediated TLS that is brought about by DNA pol III (Fujii *et al.*, 2006). The UmuD'<sub>2</sub>C also interacts with the  $\beta$  processivity clamp of DNA pol III in order to function *in vivo* (Becherel *et al.*, 2002; Sutton *et al.*, 2001).

These repair systems outlined here are dependent on control systems which respond to DNA damage. The SOS response is one such control mechanism.

## 1.7 THE SOS RESPONSE

The SOS response acts as a control mechanism in response to DNA damage. It was first characterised in *E. coli* but since then studies in other bacteria have indicated the

presence of similar systems. The response is controlled by the combined actions of the proteins LexA and RecA.

In *E.coli*, the LexA protein represses transcription of over 40 genes of the SOS regulon (Stohl, 2003; Janion, 2008). The SOS response becomes activated upon the signal of single-stranded DNA (ssDNA) (Radman, 1975). Upon DNA damage and the development of regions of ssDNA, RecA forms nucleoprotein filaments, which then act as a co-protease to enhance the expression of the SOS genes through the autocatalytic cleavage of LexA (Little, 1993, Nastri and Knight, 1994, Drees 2004a).

A SOS box is located upstream of the SOS genes, including *recA* and *lexA*. This is a palindromic sequence which LexA binds to in order to repress transcription under normal conditions, in the absence of DNA damage (Wertman and Mount., 1985). The affinities of individual SOS boxes for LexA are variable, but for key genes such as *recA* and *lexA* the affinity is weak enough to allow basal transcription (Markham *et al.*, 1984). Cleaved LexA cannot bind the SOS boxes and genes located downstream are then expressed at higher levels (Friedberg *et al.*, 1995).

The *lexA* gene itself is part of the SOS response, leading to the production of more LexA protein (Little *et al.*, 1980). This newly synthesized LexA is continuously cleaved while ssDNA is still exposed and RecA is being activated. As this continues, the genes which

have SOS boxes with higher affinities for the LexA repressor also become expressed and involved in repair. This forms a cascade of expression while the DNA damage remains present (Friedberg *et al.*, 1995). As DNA damage is repaired and the ssDNA signal is cleared up, levels of activated RecA are reduced and whole LexA is able to bind to the SOS boxes based on affinity, as LexA levels accumulate (Little *et al.*, 1980) (Figure 6).

The self-cleavage of LexA also occurs at high pH values (Little *et al.*, 1984); however this process is accelerated by activated RecA at intracellular values. The cleavage of the LexA protein occurs at a hinge region separating its two domains.

The LexA protein structure comprises a N-terminal operator binding domain at residues 1-72, a hinge at residues 70-77, followed by a beta-strand and an alpha-helix, which both interact with the rest of the C-domain (Chattopadhyaya *et al.*, 2000). Studies on the mechanism of LexA cleavage have revealed that the autocleavage site is quite close to the catalytic site. It has been shown that when pH is elevated or under the action of activated RecA, cleavage may occur following a cis to trans isomerization at the Pro 87 residue and/or a rotation of the region beta9-beta10 about beta7-beta8 following the disruption of two hydrogen bonds (Chattopadhyaya *et al.*, 2000) (See figure 29 in Appendix).

## 1.8 THE SOS RESPONSE IN MYCOBACTERIA

The *M. tuberculosis recA* gene was first isolated by the probing of the genome with the *E. coli recA* gene. It was found that the *M. tuberculosis recA* included an internal sequence of 1320bp encoding an intein which is flanked by the region homologous to *E. coli* (Davis *et al.*, 1991). To form a mature protein, the intein is excised from the precursor protein, followed by ligation of the flanking regions (Davis *et al.* 1992). The intein had previously only been found in *Saccharomyces cerevisiae* (Kane *et al.*, 1990).

A *recA* intein was also found in two other pathogenic slow growing mycobacteria, *M. bovis* and *M. leprae* out of 17 mycobacteria examined (Davis *et al.*, 1994). However the intein in *M. leprae recA* is different in sequence and location from that in *M. tuberculosis recA*

It has been suggested that the difficulties in achieving homologous recombination in the *M. tuberculosis* complex might be due to this intein structure (McFadden, 1996).

Subsequent data, however, has shown that the RecA intein does not interfere with RecA function (Frischkorn *et al.*, 1998; Papavinasasundaram *et al.*, 1997) and does not correlate with specific characteristics of different mycobacterial species such as pathogenicity or growth rate (Saves *et al.*, 2000).

The RecA proteins of *M. tuberculosis* and *M. smegmatis* are highly conserved with a 91% amino acid identity (Papavinasundaram *et al.*, 1997) (Figure 7). The *recA* gene has been successfully deleted from *M. smegmatis*, *M. bovis* BCG and *M. tuberculosis* indicating it is not essential for viability (Papavinasundaram *et al.*, 1998; Sander *et al.*, 2001) and infection in a mouse model with the *recA* mutant of *M. bovis* BCG proceeded as for wild type (Sander *et al.*, 2001). However it was found in *M. smegmatis* and *M. bovis* BCG that the  $\Delta recA$  mutant was more sensitive than the wild type to DNA damage by UV radiation, with *M. bovis* BCG also being sensitive to alkylating agents (Papavinasundaram *et al.*, 1998; Sander *et al.*, 2001).

It has been shown that the sequence upstream of the *M. tuberculosis recA* gene contains two promoter regions, P1 located closer to the *recA* gene and P2, further upstream. A mycobacterial SOS box has also been identified and is located between the -10 and -35 regions of P2 (Movahedzadeh *et al.*, 1997a).

A homologue of *E. coli* LexA has been shown to be present in mycobacteria (Movahedzadeh *et al.*, 1997b, Durbach *et al.*, 1997) and the *M. tuberculosis* LexA can bind to the upstream region of the *M. tuberculosis recA* and *lexA* genes (Movahedzadeh *et al.*, 1997a, Movahedzadeh *et al.*, 1997b). However, the *M. tuberculosis* LexA binding site or SOS box has been shown to be different from that of *E. coli* (Davis *et al.*, 2002).



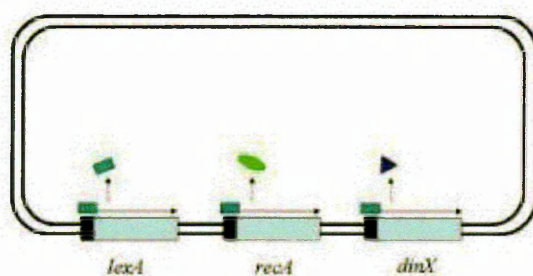
Eleven homologues of genes involved in the *E.coli* SOS response were initially found upon sequencing of the *M. tuberculosis* genome (Mizrahi and Andersen, 1998), including *recA* and *lexA* and of nine *E. coli* SOS genes examined in another study, six were shown to be induced upon DNA damage (Brooks *et al.*, 2001). It is interesting to note that two of the genes induced by DNA damage in *M. tuberculosis* were found not to have an SOS box upstream of their coding sequence, pointing to the likelihood that another mechanism, independent of LexA is utilized to activate these genes (Brooks *et al.*, 2001).

### **1.8.1 The Effect of Mitomycin C in Mycobacteria**

It has been shown in *M. tuberculosis* and *M. smegmatis* that *recA* induction can be achieved with the addition of the DNA damaging agent, mitomycin C (Movahedzadeh *et al.*, 1997a; Papavinasasundaram *et al.*, 1997; Durbach *et al.*, 1997). This is significant for research into DNA damage and repair in mycobacteria. Mitomycin C is a DNA damaging agent which acts as a bifunctional alkylating agent to create DNA lesions by the covalent linkage of two bases to form intra- and inter-strand DNA cross-links and mitomycin C mono-adducts (Goranov *et al.*, 2006). This causes significant cytotoxicity to cells (Hughes *et al.*, 1991) (Tomasz, 1995) (Champeil *et al.*, 2008) by blocking DNA replication and transcription (Zhang *et al.*, 2003; Zheng *et al.*, 2003). The repair of mitomycin C-induced damage involves multiple repair pathways such as nucleotide excision repair, homologous recombination repair and translesion synthesis repair pathways (Lee *et al.*, 2006).

**FIGURE 6: The mechanism of SOS induction**

Uninduced state



Induced state



Illustration of the uninduced and induced states of SOS induction. During the uninduced state there is only basal expression of DNA repair genes, including *lexA* and *recA*. Expression of these genes is repressed by the binding of LexA to upstream SOS boxes. DNA damage leads to the formation of ssDNA lesions with which RecA forms active nucleoprotein filaments. This leads to the autocatalytic cleavage of LexA. The cleaved LexA then no longer binds to the SOS boxes and gene expression is induced.

**FIGURE 7 : An alignment of *M. smegmatis* and *M. tuberculosis* RecA proteins.**

```

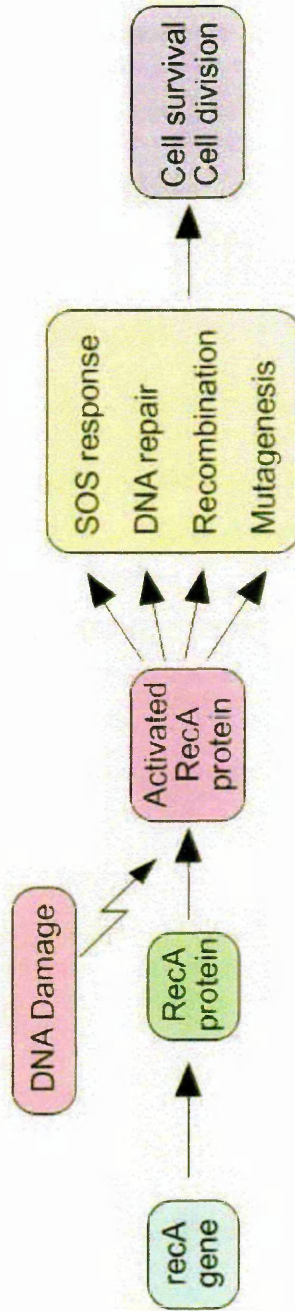
1  MAQQAPDREKALELAMAQIDKNFGKGSVMRLGEEVRQPIISVIPTGSISLD  50
   |||||...:..:|||||:|||||
1  .MTQTPDREKALELAVAQIEKSYGRKGSVMRLGDEARQPIISVIPTGSIALD  49
51  VALGIGGLPRGRVIEITGPRESSGKTTVALHAVANAQAAGGIAAFIDAHA  100
   |||||...:..:|||||:|||||
50  VALGIGGLPRGRVIEITGPRESSGKTTVALHAVANAQAAGGVAAAFIDAHA  99
101 LDPEYAKKLGVDTDSSLVSQPDGTGEQALEIADMLVRSGLDIIVIDSVAA  150
   ||:|||||...:..:|||||:|||||
100 LDPDYAKKLGVDTDSSLVSQPDGTGEQALEIADMLIRSGALDIVVIDSVAA  149
151 LVPRAEIEGEMGDSHVGLQARLMSQALRKMTGALNNSGTTAIFINQLREK  200
   ||:|||||...:..:|||||:|||||
150 LVPRAELEGEMGDSHVGLQARLMSQALRKMTGALNNSGTTAIFINQLRDK  199
201 IGVFMFGSPETTTGGKALKFYASVRLDVRRRIETLKDGTDAVGNRTRVKVVK  250
   ||:|||||...:..:|||||:|||||
200 IGVFMFGSPETTTGGKALKFYASVRMDVRRVETLKDGTNAVGNRTRVKVVK  249
251 NKVSPPFKQAEFDILYGQGISREGSLIDMGVEHGFIRKSGSWFTYEGEQL  300
   ||...:..:|||||:|||||
250 NK.SPPFKQAEFDILYGKGISREGSLIDMGVDQGLIRKSGAWFTYEGEQL  298
301 GQGKENARKFLENTDVA NEIEKKIKEKLGIGAVVTAE..DDVLPAPVD  348
   |||||...:..:|||||:|||||
299 GQGKENARNFLVENADVADEIEKKIKEKLGIGAVVTDDPSNDGVLPAPVD  348
349 F 349
   |
349 F 349

```

Alignment showing a 91% identity and 94% similarity between *M. smegmatis* and *M. tuberculosis* RecA proteins.

**Alignment showing a 91% identity and 94% similarity between *M. smegmatis* and *M. tuberculosis* RecA proteins.**

**FIGURE 8: Overview of the role of RecA to promote cell survival**



**The RecA protein is activated upon DNA damage. The activated protein plays a role in the SOS response, DNA repair, recombination repair and mutagenesis to promote cell survival and cell division**

## 1.9 RECX

RecA promotes cell survival by mediating the homologous recombinational repair of damaged DNA and induction of the SOS response to maintain the integrity of the genome (Described in detail earlier) (Figure 8).

It was found in *M. smegmatis*, *M. tuberculosis* and *M. leprae* that the *recA* gene is co-transcribed with the *recX* gene, which is located downstream of and overlapping *recA* by 32bp in *M. smegmatis* and 35bp in *M. tuberculosis* and *M. leprae*. The *M. tuberculosis* *recX* gene codes for 198 amino acids and the *M. tuberculosis* and *M. smegmatis* RecX proteins share a 60% identity at the amino acid level (Figure 9).

RT-PCR has demonstrated that the two genes are within the same transcriptional unit in *M. smegmatis* (Papavinasundaram *et al.*, 1997). The overlap of the *recX* gene with the C-terminus of *recA* suggests that RecX is translationally coupled to RecA expression (Papavinasundaram *et al.*, 1997). The exact mechanism of *recX* translation in mycobacteria is not clear. Generally however, translational coupling occurs in operons, which form a transcriptional unit and are therefore transcribed on the same RNA strand. Translational coupling can be a mechanism to control the differential expression and relative stoichiometry of the different proteins of the operon. These are usually associated with a common cellular process or pathway.

RecX was first described in *Pseudomonas aeruginosa* in 1993 as an open reading frame found downstream of *recA* (De Mot *et al.*, 1993). Since then, genes which encode RecX have been found in a wide diversity of bacteria and in some plants genomes (Lin *et al.*, 2006). RecX has been found to have a conserved location with *recA* in several bacterial genomes. This too highlights the importance of RecX for RecA activity (Stohl *et al.*, 2003). Homologues of *recX* have been found to be located downstream of or overlapping *recA* in *Thiobacillus ferrooxidans*, mycobacteria, *Streptomyces lividans*, *Xanthomonas campestris* and *Xanthomonas oryzae* (Venkatesh *et al.*, 2002; Pages *et al.*, 2003; Stohl *et al.*, 2003). In some organisms, however, such as *Neisseria gonorrhoeae* and *Bacillus subtilis*, *recX* is found elsewhere in the chromosome (Stohl *et al.*, 2003; Pages *et al.*, 2003). The *recX* gene of *E. coli* is a 166 amino acid open reading frame located between *recA* and *alaSp*. It is named *oraA* (for open reading frame between *recA* and *alaSp*) (Drees *et al.*, 2004b).

*recA* and *recX* are co-transcribed in several bacteria, again highlighting the importance of RecX for RecA activity. RT-PCR has shown that *recA* and *recX* are co-transcribed in *T. ferrooxidans*, *M. tuberculosis*, and *S. lividans* (Pages *et al.*, 2003). *X.campestris* pv. *citri* is an exception, with *recA-lexA-recX* transcribed from their own promoter in a DNA damage inducible manner, even though *recX* is located downstream of *recA* in this organism. In *Herbaspirillum seropedicae*, *recX* is located 359 bp downstream from *recA*. There is an indication of a putative operator site overlapping a probable sigma 70-dependent promoter upstream of *recA* and a transcription terminator downstream from *recX* and no obvious promoter sequence in the intergenic region (Galvao *et al.*, 2003). It

has been shown that in *E. coli*, the two genes are separated by a palindromic sequence that reduces transcriptional read-through to 5-10% (Drees *et al.*, 2004b).

RecA and RecX are both induced during the SOS response in mycobacteria (Rand *et al.*, 2003). RecX has also been found to be up-regulated during the SOS response in several bacteria, pointing to the likelihood of a regulatory role for RecX during the SOS pathway of DNA repair (Drees *et al.*, 2004a; Stohl *et al.*, 2003; Pages *et al.*, 2003).



**FIGURE 9: Alignment of *M. smegmatis* and *M. tuberculosis* RecX proteins**

```

1 MTSSRPRLTSEPSDVPDGEQAQDPRTREEQAQNVCLRLLTVRARTRAELE 50
1 .....MTVSCPPPSTSEREEQARALCLRLLTARSRTRAELA 36
51 TQLTKRGYPDDVSARVLDRLTEVGLIDDEDFAEQWVRSRHLNAGKGKRAL 100
37 GQLAKRGYPEDIGNRVLDRLAAVGLVDDTDFAEQWVQSRRANAASKRAL 86
101 AVELRKKGVDDDEVISSALADLDPAAERQRAEQLVRDKLRERLDGTPEND 150
87 AAELHARGVDDDVITTVLGGIDAGAERGRAEKLVRARLRREVLIDDGTDE 136
151 VKVTRRLVGMLARRGINQSMAYDVVSVELANERERRRV 188
137 ARVSRRVLVAMLARRGIGQTLACEVVIAELAAERERRRV 174
```

**Alignment showing a 60% identity and 67% similarity between *M. smegmatis* and *M. tuberculosis* RecX proteins**



It was shown in mycobacteria that *recX* is essential for cell viability in *M. smegmatis* when the *recA* gene is overexpressed (Papavinasundaram *et al.*, 1997; Papavinasundaram *et al.*, 1998). It was also shown in a *M. smegmatis*  $\Delta$ *recA* strain, where *recX* expression was also prevented, that even expression of RecA from its own promoter on a replicating vector was toxic (Papavinasundaram *et al.*, 1998). This toxicity was reversed when a copy of *recX* was added to the complementing plasmid (Papavinasundaram *et al.*, 1998).

Therefore *recX* acts to attenuate the deleterious effects induced by *recA* over expression, implying the role of RecX as a regulator of RecA function in mycobacteria. RecX possibly acts as a negative modulator of RecA expression or activity, to quell inappropriate recombinational repair during normal DNA metabolism in this organism.

Similar effects of RecX on RecA function have been shown in other organisms. As in the case of mycobacteria, it has been shown in *Pseudomonas aeruginosa*, *Streptomyces lividans*, and *Xanthomonas oryzae* that the over expression of homologous RecA proteins from plasmid constructs is deleterious in the absence of *recX* (Sano *et al.*, 1993; Sukchawalit *et al.*, 2001; Vierling *et al.*, 2000; Drees *et al.*, 2004b). In *Neisseria gonorrhoeae*, it was found that a *recX* mutant was deficient in all RecA-mediated processes (Stohl *et al.*, 2003; Drees *et al.*, 2004b) and exhibits decreased pilus phase variation, DNA transformation and DNA repair capacity (Pages *et al.*, 2003). Another phenotype is the 30% reduced colony size of a *recX* deletion *Streptomyces lividans* strain,

compared to the wild type (Pages *et al.*, 2003) and an increased sensitivity to UV light as well as an increased sensitivity to methyl methanesulfonate in *Herbaspirillum seropedicae* (Galvao *et al.*, 2003).

When this work was started and while it was being carried out, these various observations led to the conclusion by several authors that *recX* may play a regulatory role in *recA* function and that RecX may either have multiple activities in a particular bacterial species, or RecX may show variable activity between species.

Current knowledge about RecX includes structural details about the RecX protein, models for the mechanisms of interaction between RecA and RecX and other DNA repair proteins and various studies providing evidence for the *in vivo* and *in vitro* influence of RecX on RecA activity.

Structurally RecX has been shown to be a nine helical bundle protein consisting of two domains. It has been proposed that there is a smaller C-terminal domain, which has two amphipathic helices with a helix turn helix motif. This is stabilized by hydrophobic interactions between Phe 165, Trp 162, Phe 138, and Lys 141 (Vanlooock *et al.*, 2003) and the folding is shown to be stabilized by hydrophilic interactions between the two domains. There is no indication of super helical coils, as there is no significant crossover angle among the helices. The surface of the molecule has been calculated by the electrostatic potential surface and is mostly neutral (Mishra *et al.*, 2003). These findings

were drawn from structural studies carried out by circular dichroism, fluorescence spectroscopy, mass spectroscopy, gel permeation chromatography and structural modelling (See figure 30 in Appendix).

It has been found that RecX is involved in the blocking of RecA protein filament extension. The mechanism involves a capping of the RecA filament by RecX, at the end that extension occurs. The RecX monomers are able to bind at the interface between adjacent RecA monomers of the RecA filament, capping filament extension. While this is taking place, the end-dependent disassembly of RecA filaments continues, resulting in a net disassembly of RecA filaments and the gradual loss of RecA function (Lusetti *et al.*, 2004).

Recent research has shown that RecX is a part of a regulatory network of proteins which act on RecA recombinase. Other proteins which have been shown to modulate RecA activity are DinI and RecF (Lusetti *et al.*, 2004, 2006; Baitin *et al.*, 2008).

DinI stabilizes RecA filaments by binding to RecA protein monomers along the length of the filament. In this way, DinI is able to suppress the end-dependent disassembly action while extension of the RecA filament occurs (Lusetti *et al.*, 2004). RecX has an antagonistic effect to DinI on RecA regulation. RecX is able to prevent RecA filament extension by capping RecA and also by displacing DinI to permit filament disassembly (Lusetti *et al.*, 2004). DinI and RecX act as modulator proteins of RecA which are able to

replace each other; RecA filaments are able to change between the two forms RecA-DinI and RecA-RecX (Lusetti *et al.*, 2004).

RecF protein acts with RecO and RecR to establish RecA filaments at daughter strand gaps (Sandler and Clark, 1994; Kuzminov and Stahl, 1999; Madiraju *et al.*, 1988; Sawitzke and Stahl, 1992; Sawitzke and Stahl, 1994; Smith *et al.*, 1987; Whitby and Lloyd, 1995). At these gaps, the SSB protein usually limits the RecA filament assembly nucleation phase but enhances the extension phase. SSB removes the secondary structure of ssDNA so that it does not impede the growth of RecA filaments (Kowalczykowski and Krupp, 1987; Lavery and Kowalczykowski, 1992; Lusetti *et al.*, 2006). RecX blocks the extension phase of RecA filament extension. However, when RecF is present, RecF sequesters RecX through direct interaction and RecX is unable to inhibit filament extension (Lusetti *et al.*, 2006).

Knowledge about the network of proteins involved in the regulation of RecA activity is currently expanding. A complicated network of interactions between RecX, DinI, RecFOR, SSB and other proteins is being studied to determine how these proteins are able to regulate the RecA protein filament and the functions of RecA.

## 1.10 AIMS OF THE PROJECT

To discuss the aims of this study, it is appropriate to first introduce the hypothesis upon which it was based and through which the research was approached.

The RecA protein is key in the mycobacterial response to DNA damage and plays a significant role to promote cell survival. At the time that this work was started, recent work carried out in *Mycobacterium smegmatis* had demonstrated that the over expression of *recA* from a plasmid construct is deleterious or lethal in the absence of *recX* (Papavinasundaram *et al.*, 1997), implying the role of RecX as a regulator of RecA function. This demonstration of lethality when *recA* is over expressed in the absence of *recX* subsequently formed the foundation of further study of *recX* and its role in regulation of *recA*.

The hypothesis that RecX acts by down regulating either the expression of the *recA* gene or the activity of the RecA protein has been explored in this study.

The aims of this project were to study the role of RecX in mycobacteria by the application of various *in vivo* and *in vitro* techniques. Specific aims were as follows:

1. To identify protein-protein or protein-DNA interactions with RecA or the *recA* promoter region
2. To assess the influence of RecX on RecA ATPase activity and on RecA-stimulated LexA cleavage *in vitro*
3. To assess the consequences *in vivo* of the effect of RecX on RecA biochemical activity

## CHAPTER 2

### METHODS AND MATERIALS

#### 2.1 BACTERIAL STRAINS AND MEDIA

*E. coli* strain DH5 $\alpha$  (Sambrook *et al.*, 1989) was used as the host strain for plasmid constructions and BL21 (DE3) pLysS (Novogen Inc.) for protein expression and purification. *E. coli* cells were grown at 37°C in L-medium (Sambrook, *et al.*, 1989) containing antibiotics, where appropriate, at the following concentrations: ampicillin 50  $\mu\text{g/ml}$ ; kanamycin, 50  $\mu\text{g/ml}$ . The BL21-strains carrying plasmids for protein expression were grown in Terrific broth (12g tryptone, 24g yeast extract, 4ml glycerol, 900ml water, 100ml  $\text{KH}_2\text{PO}_4$ ,  $\text{K}_2\text{HPO}_4$ ) supplemented with 100  $\mu\text{g/ml}$  carbenicillin and 34  $\mu\text{g/ml}$  chloramphenicol. *M. smegmatis* mc<sup>2</sup>155 (Snapper *et al.*, 1990) was grown at 37°C in modified Dubos broth (Difco) or on 7H11 agar. For mycobacteria, kanamycin was used at 25  $\mu\text{g/ml}$  (20  $\mu\text{g/ml}$  in liquid medium) and gentamycin was used at 20  $\mu\text{g/ml}$ .

#### 2.2 *E. COLI* COMPETENT CELLS PREPARATION AND TRANSFORMATION

*E. coli* DH5 $\alpha$  competent cells were prepared from logarithmically growing cultures (Sambrook *et al.*, 1989). Luria broth (50 ml) was inoculated with 0.5ml of an overnight grown culture and grown to an OD<sub>600</sub> of 0.3-0.4 and cooled on ice for 10 min. The cells

were collected by centrifugation at 5000 *g* for 5 min at 4 °C, washed twice with 20 ml of ice-cold 0.1M CaCl<sub>2</sub> and finally resuspended in 5 ml of 0.1M CaCl<sub>2</sub>. Glycerol was added to a final concentration of 17% and the aliquots of cells were frozen at – 70 °C.

DNA transformation was carried out following the standard protocol, which involves the heat shock step of 42 °C for 90 seconds (Sambrook, *et al.*, 1989). The cells were incubated with L-broth when needed and plated on selection plates.

## **2.3 RECOMBINANT DNA TECHNIQUES**

### **2.3.1 PCR**

PCR reactions were carried out using the Expand high fidelity PCR system (Roche) in a 50µl reaction containing 1.25 mM MgCl<sub>2</sub>, 200µM of dNTPs, 300nM of primers, 10ng of template and 1.25 U of the enzyme. To amplify PCR products of <1kb, the PCR cycle parameters used were as follows; a first denaturation step at 94°C for 2 min, followed by 30 cycles at 94°C for 30s, annealing 60°C (or appropriate temperature) for 30s and elongation at 72°C for 1 min. PCR was concluded with a final prolonged elongation of 72°C for 7 min. PCR products were purified using the Qiaquick gel extraction kit (Qiagen).

### **2.3.2 Restriction digestion, ligation and transformation**

Restriction digestions were carried out using enzymes obtained from New England Biolabs or Roche and using recommended buffers. Digested vectors were treated with

calf alkaline phosphatase (Roche) in the digestion buffer for 1h at 37 °C. The alkaline phosphatase catalyzes the removal of 5' phosphate groups from the vector DNA. As the vector then lacks the 5' phosphoryl termini required by ligases, they cannot self-ligate. This reduces vector background following cloning.

Vectors were then eluted with Qiagen gel extraction kit either directly or after agarose gel electrophoresis. Ligation of the vector and inserts (restriction digested PCR products or plasmid fragments eluted from an agarose gel) were carried out using buffers and enzyme supplied with the Rapid DNA ligation kit (Roche). Ligations were usually carried out at room temperature for 30 min after which a part of the reaction was transformed into competent *E. coli* DH5 $\alpha$  cells. Plasmid DNA was prepared from transformants using the QIAprep spin miniprep kit (Qiagen).

### **2.3.3 Automated sequencing**

This was used to verify the proper construction of cloned plasmids such as those produced and used for the yeast two-hybrid assay (section 2.7), the recombination assay (section 2.11), viability assays (section 2.10) and the effect of RecX on RecA levels (section 2.13). The nucleotide sequence of PCR amplified genes and the cloning junctions in the plasmids were determined using d-Rhodamine terminator cycle sequencing ready reaction kit (Applied Biosystems) containing 5% DMSO, 500 ng template and 3.2pmol of the appropriate primer. The cycle sequencing parameters consisted of 25 cycles of denaturation at 96°C for 30 s, annealing at 60°C for 15 s and elongation at 60°C for 4 min. After the cycle sequencing reaction, the DNA was



precipitated with 74µl of 70% ethanol/ 0.5mM magnesium chloride and then processed through automated sequencing in a 377 ABI PRISM sequencing machine.

## **2.4 PROTEIN EXPRESSION**

### **2.4.1 Protein expression in *E. coli***

Plasmids were transformed into *E. coli* strain BL21 (DE3) pLysS (Novogen Inc) by the standard transformation protocol described earlier. The transformants were grown to an OD<sub>600</sub> of 0.6 in L-broth supplemented with carbenicillin and chloramphenicol and induced with 1mM IPTG at 37°C for 3 h.

### **2.4.2 Purification of RecX**

A 3 litre culture of the BL21 (DE3) pLysS (Novogen Inc.) strain carrying the RecX expression construct pSM1 (Table 2) was grown at 37°C and induced with 1mM IPTG when the OD<sub>600</sub> of the culture reached 0.8. The culture was allowed to grow for a further 6 h at 30°C in a shaker at 300rpm and then harvested. Cells were lysed by sonication in a lysis buffer containing 50mM phosphate buffer pH8.0, 10mM Tris, 200mM NaCl and 8M urea. Cells were then spun at 9000 rpm on a Jasco JA14 rotor for 20 min and the pellet was discarded. The supernatant was then put through centrifugation at 20,000 rpm on a JA 25.5 rotor for 20 min. The supernatant was then allowed to bind to 5ml of Talon resin (Clonetech Inc.) pre-equilibrated with the lysis buffer. The slurry was allowed to tumble in the cold room for 1 h. After a low speed spin of 5,000 rpm for 20 min, the

Talon resin was resuspended in a small amount of lysis buffer and packed into a FPLC column (Pharmacia Inc.). The protein was bound at pH 8.0 and eluted at pH 6.0. Buffer compositions were 50mM Tris, 200mM NaCl, 6M urea, or 4M urea or 2M urea, or not including urea. The protein was purified further by size exclusion chromatography on a Superdex S-75 column (Pharmacia Inc.). The purified *M. tuberculosis* RecX protein was visualised following electrophoresis through a SDS-PAGE (15% polyacrylamide) gel.

## **2.5 GEL SHIFT ASSAY TO DETERMINE IF RECX BINDS TO THE *RECA* PROMOTER REGION.**

### **2.5.1 PCR amplification of *recA* promoter regions**

The *recA* upstream regions were amplified by PCR. The *M. smegmatis* *recA* upstream region, 609bp including 80bp of the *recA* gene, was amplified from DNA template pKP68 (Table 2) using primers p106 and p107 (Table 3), with an annealing temperature of 64°C. The *M. tuberculosis* *recA* promoter region, 532bp including 93bp of the *recA* gene, was amplified from DNA template pKP77 (Table 2) using primers p108 and p109 (Table 3) with an annealing temperature of 66°C. The PCR reactions were set up as described earlier.

### **2.5.2 DNA labelling, the binding reaction and electrophoresis**

The DNA was labelled using 5pmol of DNA, 200U of T4 PNK enzyme and  $\gamma$ -<sup>32</sup>P ATP, at 37°C for 10 min. The double-stranded oligonucleotides were diluted to 30fM with

binding buffer (HEPES 20mM pH 7.9, EDTA 1mM, DTT 1mM, Tween20 1%, KCl 30mM, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 10mM), with an addition of 2µg poly (dI-dC) and poly L lysine. The binding reaction was carried out for LexA, with each DNA sequence, using 100ng of LexA per reaction. The same was done for RecX (purified as described above) with 918ng, and three subsequent ten-fold dilutions. Following incubation at room temperature for 15min, the samples were applied to a pre-electrophoresed native 8% (w/v) polyacrylamide gel. The radioactive bands were visualised by exposure of the dried gel to X-ray film.

## **2.6 LEXA CLEAVAGE ASSAYS**

### **2.6.1 RecA Activation**

10µM of *M. tuberculosis* RecA (purified by another lab) was activated in reaction buffer (20mM Tris, 2mM MgCl<sub>2</sub>, pH 7.4) (to final concentrations), 5mM DTT, 320ng/ml calf thymus DNA (made single stranded by boiling for 5 minutes and rapidly cooling on ice) and 10mM ATPγS, in a total volume of 20µl and added together in this specific order. Tubes were also prepared by adding *M. tuberculosis* RecX which was 1/3, 1/2, equimolar, 2x and 3x the amount of RecA. RecX was added immediately after RecA unless otherwise indicated. Samples were incubated at 0°C for 45 min.

### 2.6.2 LexA cleavage

After activation, the activated samples were added to tubes, which were pre-incubated at 37°C, containing 60µM LexA (New England Biolabs) in reaction buffer. The tubes were incubated at 37°C for 1 h to allow LexA cleavage to occur. At the end of the cleavage reactions, sample buffer was added and the tube was rapidly cooled on ethanol-dry ice and frozen at -70 °C. The products were resolved by electrophoresis through a 15% polyacrylamide SDS-gel and the bands were viewed following staining with the Rapid-Coomassie stain (Sambrook, *et al.*, 1989).

Control experiments to test for specificity were carried out with bovine serum albumin (BSA) (obtained commercially), NusB (a transcription antitermination protein purified within the division) from *M. tuberculosis*, S10 (a small ribosomal subunit protein purified within the division) from *M. tuberculosis* and calcium binding protein (CBP) (obtained commercially) from *Entamoeba histolytica* at concentrations 3x that of RecA.

### 2.6.3 pH-dependent autocleavage of LexA

pH-dependent autocleavage of LexA was investigated by incubating 20µM LexA (New England Biolabs) in a 0.5M CAPS buffer pH10 containing 0.5M NaCl at 37°C for 24 h. RecX (8.4µM) (purified as described above) was included in this mix and the effect on LexA cleavage assessed. Incubations at 4°C served as controls.

## 2.7 YEAST-2-HYBRID ASSAY

A forward yeast-two hybrid assay was carried out to investigate interactions between RecA and RecX *in vivo*. This was done by looking for interactions between proteins bound to the activating and binding domains of the GAL4 transcriptional activator protein of the yeast *Saccharomyces cerevisiae*. Activity is detected via the activation of expression of reporter genes (Fields and Song, 1989).

The *Saccharomyces cerevisiae* strain GC1945 (CLONTECH) which was used throughout this yeast two-hybrid analysis. The 'bait' and 'prey' fusions were constructed in vectors pGBD-C2 and pGAD-C2 respectively, as suggested by James *et al* (1996).

The *M. tuberculosis recX* gene was cloned from plasmid pSM1 (Methods table 2) as a *NdeI* - *BamHI* fragment between the *SmaI* and *BglII* sites of both pGBD-C2 and pGAD-C2.

The *M. tuberculosis recA* gene was isolated from plasmid pKP77 (Methods table 2), and cloned into the *SmaI*-*BglII* sites of the same vectors.

Selected clones of RecA-PGAD, RecA-PGBD, RecX-PGAD and RecX-PGBD were sequenced using primers F34807 and F34808 (Methods, table 3) and checked before being applied to the assay.

The clones were transformed into *S. cerevisiae* cells in the following combinations:

1. PGBD+PGAD
2. PGBD+PGAD-RecA
3. PGBD+PGAD-RecX
4. PGBD-RecA+PGAD
5. PGBD-RecA+PGAD-RecA
6. PGBD-RecA+PGAD-RecX
7. PGBD-RecX+PGAD
8. PGBD-RecX+PGAD-RecA
9. PGBD-RecX+PGAD-RecX
10. +ve control (A standard construct used as a positive control test by the lab for this assay called 15/17 NusE).

The transformants were selected on leu-, trp-, his<sup>+</sup> plates (based on markers in each plasmid). These were then tested for the activation of reporter genes by the colony-lift filter assay using X-Gal. When there is an interaction, dark blue colonies are produced on plates containing X-Gal. The *S. cerevisiae* cells containing the interacting hybrid proteins can be detected easily against a background of cells lacking such an interaction as these are white, indicating that the RecA and RecX proteins interact directly *in vivo*.

## **2.8 COMPLEMENTATION OF THE HS42-DELETION MUTANT**

### **2.8.1 Preparation of electrocompetent cells**

The *M. smegmatis* *recA* deletion mutant (HS42) (Papavinsasundaram *et al.*, 1998) was grown in Dubos medium (Dubos broth 240ml, Dubos Albumin 10ml, 0.2% Glycerol) until the culture reached an OD<sub>600</sub> of 1.0. This strain has also had removed part of *recX* (so RecX would not be present) as well as all of *recA* along with its promoters.

The cells were harvested by centrifugation at 5,000 g for 10 min using a HS4 rotor at 4°C. Cells were washed four times in 10% glycerol and finally suspended in 1/10 culture volume at 4°C.

### **2.8.2 Transformation by electroporation**

Transformation was carried out using a Bio-Rad Gene pulser. Electroporation was carried out using 100ng DNA and 0.4ml of competent cells, in ice-cold electroporation cuvettes (0.2cm cuvette, Bio-Rad), with a pulse of 25µF, 2.5kV, 1000Ω. The electroporated cells were transferred to 4.6ml of Dubos medium and incubated at 37°C for 2 h. 100µl of the culture was then plated on 7H11 plates with hygromycin and kanamycin.

## **2.9 WESTERN ANALYSIS TO VERIFY RECA EXPRESSION**

The *M. smegmatis* strains were grown in 100ml of Dubos broth and treated with 0.2 µg/ml of mitomycin C for 5 h. The optimisation for *M.smegmatis* is described in

Movahedzadeh *et al.* (1997a). The cells were spun down, washed in 1 X PBS. Alternatively they were washed with 1x Z buffer (0.06M Na<sub>2</sub>HPO<sub>4</sub>, 0.04M NaH<sub>2</sub>PO<sub>4</sub>.2H<sub>2</sub>O, 0.01M KCl, 1mM MgSO<sub>4</sub>.7H<sub>2</sub>O, made to pH 7.0) (Papavinasundaram *et al.*, 2001). The cells were resuspended in 0.5ml and lysed using glass beads in a ribolyser (Papavinasundaram, *et al.*, 1997). 20µg of the cell free extracts, as determined by the BCA protein assay kit (Pierce), were separated by SDS-polyacrylamide gel electrophoresis through a 10% gel. The proteins were then electroblotted onto a PVDF membrane (Immobilon-P, Millipore). The membrane was blocked with 10% non-fat milk in TTBS (20mM Tris, pH 7.5, 0.5M NaCl buffer containing 0.1% Tween 20) and reacted with anti-*M. tuberculosis* RecA antiserum (1:250 dilution). The RecA antiserum was raised in mice against recombinant *M. tuberculosis* RecA protein and was produced in the division. Anti-mouse antibody conjugated to horseradish peroxidase (Dako) was used as the second antibody. After washing with TTBS, the blot was developed with diaminobenzidine reagent solution (Davis *et al.*, 1992).

## 2.10 VIABILITY ASSAY

This assay was carried out for HS42-complemented with pKP107 and pKP108 (Table 2) (Figure 10). The four strains were grown in 100ml Dubos broth with hygromycin, kanamycin and 0.1% Tween 20. Cultures were incubated at 37°C and shaken at 300 rpm until OD<sub>600</sub> of 0.6, when the culture was divided into two flasks, with 50ml of culture in each. One culture was treated with 0.2µg/ml mitomycin C. Both flasks were then incubated as before for 5 h. After 5 h 3 aliquots were taken from each flask, induced and



uninduced. Each aliquot was serially diluted from  $10^{-1}$  to  $10^{-6}$  by diluting 50 $\mu$ l of culture in 450 $\mu$ l of 10% glycerol with 0.1% Tween 20. 50 $\mu$ l of each dilution was plated on 7H11 plates with hygromycin and kanamycin and incubated at 37°C. Colony numbers were counted after 3 days.

**FIGURE 10: Viability Experiments with the Deletion  
Mutant-Complemented Strains of  
*M. smegmatis***

**Strains:**



*M. smegmatis* *recA*



*M. smegmatis* *recA + recX*



Use 3 colonies of each strain.



Grow in liquid broth until OD 0.6.  
Induce for 5 hours with mitomycin C



Take 3 aliquots.  
Plate serial dilutions of  $10^{-1}$  to  $10^{-6}$   
of induced and uninduced cultures.



Count colonies and compare survival numbers.

**Summary of a viability assay carried out to investigate the effect of RecX on cell survival following DNA damage. The assay was carried out using a *M. smegmatis* *recA-recX* deletion mutant complemented with either *recA* or *recA+recX*. Cultures of were treated with mitomycin C to induce DNA damage and numbers for cell survival were compared.**

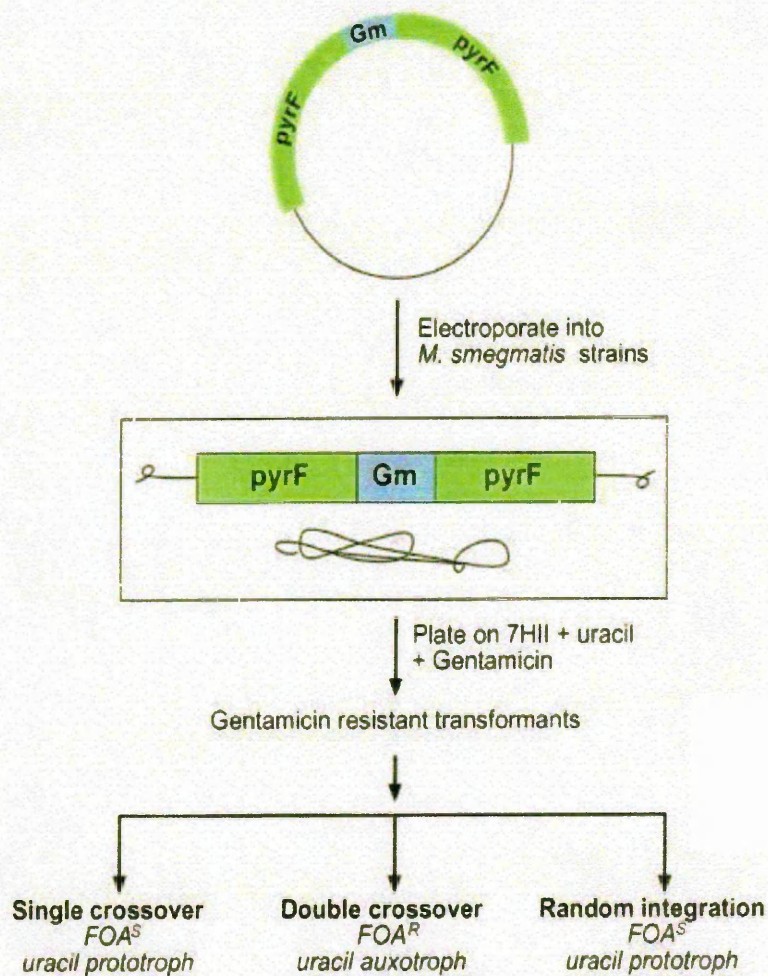
## 2.11 HOMOLOGOUS RECOMBINATION ASSAY

A homologous recombination assay was carried out to study numbers of double crossover events in the *M. smegmatis* *recA* (pKP 107) and *recA+recX* (pKP 108) complemented strains of the HS42 deletion mutant (Table 2) (Figure 11)

The *pyrF* gene, disrupted by a gentamycin resistance cassette in a non-replicating vector was introduced into the *M. smegmatis* cells. If the resistance gene integrates into the chromosome then gentamycin resistant colonies could arise by double crossovers involving the replacement of the wild type *pyrF* gene with the disrupted allele, which gives resistance to fluoro-ototic acid (FOA) (5-fluorouracil-6-carboxylic acid monohydrate) and uracil auxotrophy. Single crossovers and random integrations result in integration of the whole plasmid and both the wild type and mutated alleles are present, therefore they remain uracil prototrophs are sensitive to fluoro-ototic acid.

Competent cells were produced as described above. These were electroporated with the *pyrF* targeting suicide plasmid pKP134 (Table 2) as described above. A positive control was done with plasmid pKP121. After transformation, 3ml of cells were spun down and plated on 7H11 with gentamycin (20µg/ml) and uracil (0.2mM). Colonies were transferred by replica plating to 3 plates: 7H11 with gentamycin and uracil, 7H11 with gentamycin and 7H11 with gentamycin and fluoro-ototic acid (1mg/ml). These were incubated at 37°C and numbers of colonies counted after three days.

**FIGURE 11: Recombination Assay to Study the Numbers of Double Crossover Events in the Different Complemented Strains:**



Summary of a recombination assay carried out to study the role of RecX on homologous recombination by looking at numbers of double crossovers that occur in the *M. smegmatis* *recA-recX* deletion mutant complemented with *recA* or *recA+recX*. These were electroporated with the *pyrF* targeting suicide plasmid pkp134. Cells were plated on 7H11 with gentamycin and uracil, 7H11 with gentamycin and 7H11 with gentamycin and fluoro-orotic acid and numbers of colonies counted after growth

## 2.12 ATPASE ASSAYS

ATPase assays were carried out in order to study the process of ATP hydrolysis by *M. tuberculosis* RecA and the effect of *M. tuberculosis* RecX on this reaction (Weinstock *et al.*, 1981, Muniyappa *et al.*, 1996).

### 2.12.1 RecA activation

A reaction mix was set up containing 35mM Tris HCl pH 7.5, 90µg/ml BSA, 2mM MgCl<sub>2</sub>, 1.5mM DTT, 1µM *M. tuberculosis* RecA, 100µM non-radioactive ATP, 2.5µl of [ $\alpha$ -<sup>32</sup>P] dATP redivue deoxyadenosine 5'  $\alpha$ -<sup>32</sup>P – triphosphate triethylammonium salt (Amersham AA0004) and 32µg/ml calf thymus DNA which was added to the reaction mix after it was made single stranded by boiling and storing on ice.

The reaction mix was made up to a volume of 50µl and then divided into aliquots of 5µl each. A non-DNA control reaction mix was prepared using the same concentrations as above but without the DNA and up to a volume of 10µl. This was then divided into two aliquots of 5µl each.

### 2.12.2 ATP hydrolysis

The aliquots were incubated at 37°C for 30min in a water bath. Individual aliquots were removed at time points of 0, 2, 4, 6, 8, 10, 15, 20 and 30 min and aliquots for the non-DNA controls were removed at 15 and 30 min. As soon as a sample was removed from

the water bath at a specific time point, the reaction was stopped by the addition of 2 $\mu$ l of a mixture containing 3mM ADP, 3mM non-radioactive ATP and 25mM EDTA for every 5 $\mu$ l of the reaction mix.

### **2.12.3 Thin layer chromatography**

The samples for the individual time points were then separated by thin layer chromatography. The samples were spotted on 8cm by 6cm polyethyleneimine cellulose strips. Each sample was loaded to be 1cm apart and 1cm from the bottom of the strip. An ATP standard was also loaded on to the strip.

The strip was then placed in a glass jar lined with 3M paper. 0.5M NaH<sub>2</sub>PO<sub>4</sub> developing solution was then poured into the jar up to a depth of 1cm. The strip was removed when the solution had travelled all the way up and it was then allowed to air dry. After the strip was dry, it was developed and viewed using a phosphoimager.

### **2.12.4 Data analysis**

A volume analysis was then carried out on the radioactive spots to calculate the percentage ATP hydrolysis over time. This was done using the Image quant programme. Mean values were calculated for three sets of experiments and the results of the analysis were plotted on a graph showing the mean rate of ADP produced over time.

### 2.12.5 Experiment variations

The experiments were repeated with the addition of *M. tuberculosis* RecX at a concentration of 3 $\mu$ M. Therefore the RecA and RecX were at a ratio of 1:3. This was to determine if RecX has an effect on ATP hydrolysis by RecA.

The experiments were also repeated using Calcium binding protein from *Entamoeba histolytica* at a concentration of 3 $\mu$ M. This was a control experiment to look at specificity of RecX.

The experiments were also repeated using *M. tuberculosis* RecX at a concentration of 1 $\mu$ M and therefore the ratio of RecA: RecX was 1:1.

The experiments were also repeated using RecX at a concentration of 3 $\mu$ M, with the addition of RecX before the activation of RecA by DNA. This experiment was the same as described previously except that the RecX was added to the reaction mix before the reaction mix had been on ice for 45min.

Each experiment set was repeated three times and the mean values were plotted on a graph as described earlier, for comparison.

## **2.13 TIME-COURSE EXPERIMENTS TO DETERMINE THE EFFECT OF RECX ON THE RECOVERY LEVELS OF RECA FOLLOWING PULSE DNA DAMAGE.**

### **2.13.1 DNA damage induction and harvests**

This experiment was carried out for the HS42-complemented strains pKP107 and pKP108 (Table 2). 500ml of each of the cultures were grown up to an OD of 0.6 and then divided into two separate flasks. Mitomycin C was added to one flask to a concentration of 0.2 $\mu$ g/ml. The other flask was untreated. 50 ml of the induced and the uninduced cultures were harvested. This was taken as time point 0h.

To harvest the cultures, they were spun in a centrifuge at 8,000g for 15 min at 4°C. The supernatant was then removed. 50ml of Z buffer was then added and pellet resuspended. The samples were then spun at 8000g for 15min. The pellet was then resuspended in 1ml of Z buffer and transferred to an eppendorf tube. It was spun at 12,000g for 1 min and the supernatant was removed. It was then spun again at 12,000g for 1 min and the excess supernatant was removed. The sample and the pellet was stored at -70°C.

After 1 h 200ml of both cultures were washed with Dubos medium (spun at 8,000g, for 15min at 4°C) and 3 washes in eppendorfs (2min) (as was done with Z buffer: see above), washing out all the mitomycin C.



The pellets were then resuspended in 200ml Dubos medium in fresh flasks. At 6, 24, 48 and 72 h time points after washing out the mitomycin C, a 50ml harvest for each flask was carried out.

### **2.13.2 Cell free extract production and western analysis**

The pellets were used to produce cell free extracts to use in western blots (as described previously). BCA assays were carried out to determine the protein concentrations of the samples. The samples were checked on a SDS-PAGE gel to verify concentrations and check for degradation. 15µg of each sample was then used for the western blots. These were done using anti-RecA antibodies and anti- mouse antibodies to a dilution of 1:250. The band intensities for the different strains were then viewed and compared.

**TABLE 1: LIST OF STRAINS**

| Strain                      | Relevant characteristics/ Genotype   | Reference                             |
|-----------------------------|--|---------------------------------------|
| <i>E. coli</i> DH5 $\alpha$ | <i>E. coli</i> cells used for the efficient transformation of plasmids.<br>F <sup>-</sup> , $\phi$ 80dlacZ $\Delta$ M15, $\Delta$ (lacZYA-argF)U169, <i>deoR</i> , <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> (rk <sup>-</sup> , mk <sup>+</sup> ), <i>phoA</i> , <i>supE44</i> , $\lambda$ , <i>thi-1</i> , <i>gyrA96</i> , <i>relA1</i> | Sambrook <i>et al.</i> , 1989         |
| BL21(DE3) pLysS             | <i>E. coli</i> cells used for protein expression and purification.<br>F <sup>-</sup> , <i>ompT</i> , <i>hsdS<sub>B</sub></i> (r <sub>B</sub> <sup>-</sup> , m <sub>B</sub> <sup>-</sup> ), <i>dcm</i> , <i>gal</i> , $\lambda$ (DE3), pLysS, Cm <sup>r</sup> .   | Novagen Inc.                          |
| GC1945                      | <i>Saccharomyces cerevisiae</i> strain used for yeast two-hybrid assay. It comprises of a <i>lacZ</i> gene, expression of which is controlled by the GAL4 protein which acts as a transcriptional activator. Prey and bait vectors are transformed into these cells and tested for expression.   | Fields and Song, 1989                 |
| mc <sup>2</sup> 155         | <i>M. smegmatis</i> cells used for the efficient transformation of plasmids.<br><i>recA</i> <sup>+</sup> , Hyg <sup>r</sup>  | Snapper <i>et al.</i> , 1990          |
| HS42                        | A <i>M. smegmatis</i> <i>recA</i> deletion mutant previously isolated by homologous recombination using a <i>sacB</i> counterselection strategy described in detail in and carried out by Papavinasundaram <i>et al.</i> , 1998.<br>$\Delta$ <i>recA</i> , Hyg <sup>r</sup> , Suc <sup>r</sup> .   | Papavinasundaram <i>et al.</i> , 1998 |

**Table 2: LIST OF PLASMIDS**

| <i>Plasmid</i> | <i>Construction and relevant characteristics</i>   | <i>Reference</i>                       |
|----------------|--|--|
| pGAD           | Plasmid containing the activating domain of the GAL4 protein   | Fields and Song, 1989                  |
| pGBD           | Plasmid containing the binding domain of the GAL4 protein  | Fields and Song, 1989                  |
| pHRM-C         | Non-replicating vector carrying the <i>M. smegmatis</i> <i>pyrF</i> gene disrupted by an <i>aph</i> cassette   | Sander <i>et al.</i> , 1995            |
| pKP38          | 3kb <i>AccI</i> fragment of pRISM1 made blunt-ended into <i>SmaI</i> site of pUC19   | Papavinasundaram, <i>et al.</i> , 1997 |
| pKP 65         | 1.7kb <i>PstI</i> fragment of pRISM1 into <i>PstI</i> site of pUC 19   | Papavinasundaram, <i>et al.</i> , 1997 |
| pKP 68         | <i>M. smegmatis</i> <i>recA</i> clone with flanking DNA. 1.5kb <i>EcoRI</i> - <i>Bgl</i> III fragment of pKP65 into 5.3kb <i>EcoRI</i> - <i>Bgl</i> III fragment of pKP38  | Papavinasundaram, <i>et al.</i> , 1997 |
| pKP 77         | <i>M. tuberculosis</i> <i>recA</i> clone with flanking DNA   |  |
| pKP107         | <i>M. smegmatis</i> <i>recA</i> expressed from its own promoter in integrating vector pMV306   | Papavinasundaram, <i>et al.</i> , 1998 |
| pKP108         | <i>M. smegmatis</i> <i>recA</i> + <i>M. smegmatis</i> <i>recX</i> expressed from the <i>recA</i> promoter in integrating vector pMV306   | Papavinasundaram, <i>et al.</i> , 1998 |
| pKP 121        | A pBluescript KS-based plasmid carrying a gentamycin cassette at the <i>KpnI</i> site and a mycobacterial origin of replication (1.9kb <i>NotI</i> fragment containing the <i>oriM</i> of pMV261) at <i>NotI</i> site. | Papavinasundaram, <i>et al.</i> , 1998 |
| pKP134         | <i>M. smegmatis</i> <i>pyrF</i> gene disrupted by a gentamycin cassette. 0.86kb <i>Bam</i> HI fragment of pUCGM replaced the 1.4kb <i>aph</i> cassette disrupting the <i>pyrF</i> in pHRM-C                            | Papavinasundaram, <i>et al.</i> , 1998 |
| pMV261         | Mycobacterial replicating vector carrying the BCG <i>hsp60</i> promoter  | Papavinasundaram, <i>et al.</i> , 1997 |
| pMV306         | Mycobacterial integrating vector, which integrates at a mycobacteriophage phage L5 attachment site   | Stover <i>et al.</i> , 1991            |
| pRISM1         | 8kb <i>EcoRI</i> fragment of <i>M. smegmatis</i> DNA containing <i>recA</i> in pUC9-2  | Papavinasundaram, <i>et al.</i> , 1997 |
| PSM1           | <i>M. tuberculosis</i> <i>recX</i> in pET15b vector PCR amplified from pKP77 using primers P98 and P99 and cloned between <i>NdeI</i> and <i>Bam</i> HI sites in pET15b.   |  |
| pUC19          | Described by Yanisch-Perron <i>et al</i> (1985)  | Yanisch-Perron <i>et al.</i> , 1985    |
| pUC9-2         | Described by Hanna <i>et al</i> (1984)   | Hanna <i>et al.</i> , 1984             |
| pUCGM          | Source for the gentamycin resistance gene  | Schweizer, 1993                        |

**Table 3: LIST OF PRIMERS**

| Primer             | Position   | Oligonucleotide sequence (5'-3')     |
|--------------------|--|--------------------------------------|
| F34807             | pGBD sequencing primer, to look at the cloning junction.                     | TCCTCGAGAAGACCTTGACAT<br>GATTTTGA    |
| F34808             | pGAD sequencing primer, to look at the cloning junction.                     | TTCTCAAGCGCTTTCACAACC<br>AAT         |
| F28705             | <i>M. tuberculosis</i> <i>pyrF</i> up primer.                                | CAAAGGTACCCGAAGGCCGG<br>CTCTACGACAT  |
| F28706             | <i>M. tuberculosis</i> <i>pyrF</i> down primer                               | CAAAGGTAGCCGACGACTGG<br>TGCCCGACG    |
| P98                | <i>Nde</i> -upper, <i>M. tuberculosis</i> <i>recX</i> , 4943-62 of pKP77     | TTCCTTACATATGACGGTGTCC<br>TGCCCGCC   |
| P99                | <i>Bam</i> HI-lower, <i>M. tuberculosis</i> <i>recX</i> , 5531-5510 of pKP77 | TTTAGGATCCACCCGGCAAGT<br>ATCTCCAGTTT |
| P106               | <i>M. smegmatis</i> <i>recA</i> promoter, up primer, 742-765 of pKP68        | TGAGTTCGGCACCCGCGGTCA<br>CCT         |
| P107               | <i>M. smegmatis</i> <i>recA</i> promoter, down primer, 1350-1327 of pKP68.   | CGAGCCTTTGCCGAAATTCTT<br>GTC         |
| P108               | <i>M. tuberculosis</i> <i>recA</i> promoter, up primer, 2167-2189 of pKP77   | CGCGACGTCTGGCGGGCATTG<br>TG          |
| P109               | <i>M. tuberculosis</i> <i>recA</i> promoter, down primer, 2698-2675 of pKP77 | CGCCGAGGCGCATCACCGAA<br>CCTT         |
| TB-RECX-RT-PCR-UP  | Amplification of <i>M. tuberculosis</i> <i>recX</i>                          | GCACCCGCGCCGAGTTAG                   |
| TB-RECX-RT-PCR-LOW | Amplification of <i>M. tuberculosis</i> <i>recX</i>                          | GGCCAGCCGATCCAATACCC                 |

## **CHAPTER 3**

### **RESULTS**

#### **AN ANALYSIS OF THE MODE OF INTERACTION OF MYCOBACTERIAL RECA AND RECX**

##### **3.1 AIMS**

These experiments aim to investigate the hypothesis that RecX acts by down regulating either the expression of *recA* or the activity of the RecA protein. Investigations were carried out to study the nature of the interaction between RecA and RecX.

These investigations were carried out by two approaches. One approach used was the application of the forward yeast-two hybrid assay to investigate whether a protein-protein interaction occurs between RecX and RecA *in vivo*.

The second approach involved the application of an *in vitro* assay, the DNA mobility shift assay, with the aim to investigate whether RecX binds to the *recA* upstream region to modulate *recA* expression by means of transcriptional control.

### 3.2 RECA INTERACTS WITH RECX *IN VIVO*.

A forward yeast-two hybrid assay was carried out to determine if a protein-protein interaction occurs between RecA and RecX *in vivo*.

This involved the application of the GAL4 protein of the yeast *Saccharomyces cerevisiae*, which acts as a transcriptional activator and is needed for the expression of genes of enzymes involved in galactose utilisation. The GAL4 protein is comprised of two domains, the N- and C- terminal domains. The N-terminal domain is the DNA binding domain, which binds to specific DNA sequences and the C-terminal domain, is the activating domain, which contains regions necessary to activate transcription. The assay is based on the fact that the activation function of the separately expressed domains can be restored when the DNA-binding domain and the transcription-activation domain are brought together by two interacting, heterologous proteins (Fields and Song, 1989). Activity is detected via the activation of expression of reporter genes.

Therefore when one protein is fused to the DNA binding domain and another to the activating domain and these clones are then introduced into a yeast strain in which expression of the reporter gene *lacZ* is controlled by GAL4, the colonies producing  $\beta$ -galactosidase indicate an interaction between the two proteins (Fields and Song, 1989). One such strain is *Saccharomyces cerevisiae* strain GC1945 (CLONTECH) which was used throughout this yeast two-hybrid analysis.

The DNA binding domain is generally known as the 'bait' and the activating domain as the 'prey'. The 'bait' and 'prey' fusions were constructed in vectors pGBD-C2 and pGAD-C2 respectively, as suggested by James *et al* (1996). The *recX* gene was isolated from plasmid pSM1 (Methods, Table 2) and cloned between the *Sma*I and *Bgl*II sites of both pGBD-C2 and pGAD-C2. The *recA* gene was isolated from plasmid pKP69 and cloned into the *Sma*I-*Bgl*II sites of the same vectors. Selected clones of RecA-PGAD, RecA-PGBD, RecX-PGAD and RecX-PGBD were sequenced and checked for proper insertion before being applied to the assay.

The clones were transformed into *S. cerevisiae* cells in the following combinations: PGBD+PGAD, PGBD+PGAD-RecA, PGBD+PGAD-RecX, PGBD-RecA+PGAD, PGBD-RecA+PGAD-RecA, PGBD-RecA+PGAD-RecX, PGBD-RecX+PGAD, PGBD-RecX+PGAD-RecA, PGBD-RecX+PGAD-RecX, +ve control.

The transformants were tested for the activation of reporter genes by the colony-lift filter assay using Xgal.

Positive results were observed for the following combinations (figure 12):

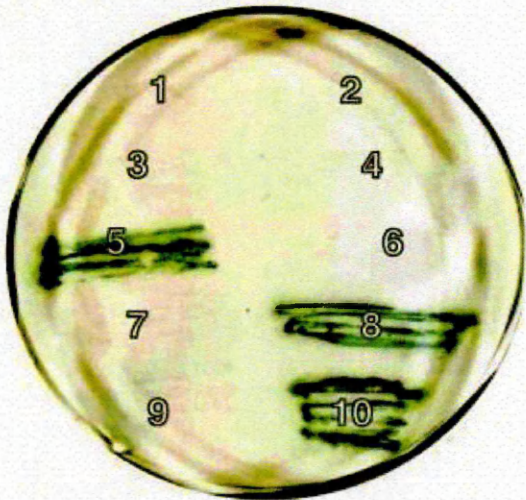
PGBD RecA + PGAD-RecA, PGBD-RecX + PGAD-RecA, positive control

The positive interaction observed for RecA and RecX suggests that these two proteins interact *in vivo*. The positive interaction observed for RecA with RecA is consistent with the fact that RecA forms complexes with itself. There was also no interaction observed for PGBD-RecA + PGAD-RecX (see summary below for explanation). Because positive

results were observed in one orientation and not the other, Western blots were carried out for the PGBD-RecA+ PGAD-RecX cells, with antibodies to the DNA binding domain and the activation domain (obtained commercially) to check for proper expression. However the blots gave inconclusive results.



**FIGURE 12: Yeast Two-Hybrid Assay showing Protein-Protein Interaction between RecA and RecX**



| Colony No. | Combination               | Interaction |
|------------|---------------------------|-------------|
| 1          | pGBD vector + pGAD vector | -           |
| 2          | pGBD vector + pGAD RecA   | -           |
| 3          | pGBD vector + pGAD RecX   | -           |
| 4          | pGBD RecA + pGAD vector   | -           |
| 5          | pGBD RecA + pGAD RecA     | +           |
| 6          | pGBD RecA + pGAD RecX     | -           |
| 7          | pGBD RecX + pGAD vector   | -           |
| 8          | pGBD RecX + pGAD RecA     | +           |
| 9          | pGBD RecX + pGAD RecX     | -           |
| 10         | Positive control          | +           |

*Saccharomyces cerevisiae* strain GC1945 cells were transformed with pGBD (vector with DNA binding domain) and pGAD (vector with DNA activating domain), containing *recA* or *recX* in various combinations (see above for combinations used). The transformants were tested for activation of reporter genes by means of the filter lift X-gal assay. When there is an interaction, dark blue colonies are produced on plates containing X-gal. The *S. cerevisiae* cells containing the interacting hybrid proteins were detected easily against a background of cells lacking such an interaction as these are white. Positive interactions were observed for pGBD RecA + pGAD RecA and pGBD RecX + pGAD-RecA, indicating that the RecA and RecX proteins interact directly *in vivo* .

### **3.3 THE RECX PROTEIN DOES NOT REGULATE *RECA* EXPRESSION OF *M. TUBERCULOSIS* OR *M. SMEGMATIS* BY TRANSCRIPTIONAL CONTROL.**

#### **3.3.1 Purification of *M. tuberculosis* RecX Protein.**

The *M. tuberculosis* *recX* gene was cloned into the expression vector pET15b introducing a N-terminal His-tag, and the resulting plasmid, pSM1 (Methods Table 2), was transformed into *E. coli* BL21(DE3) pLysS (Novogen Inc) cells. Protein expression was induced with 1mM IPTG at 37°C for 3 hours. The *M. tuberculosis* RecX protein was then purified by means of the His-tag using immobilized metal affinity chromatography (IMAC), followed by size exclusion chromatography. The purified 22kDa protein was visualised following electrophoresis through a SDS-PAGE (15% polyacrylamide) gel and run alongside low range prestained SDS-PAGE standards (Bio-rad). The gel was stained with Coomassie blue (Figure 13).

#### **3.3.2 DNA mobility shift assay showing no interaction between RecX and the mycobacterial upstream regions.**

Following purification, the RecX protein was used in DNA mobility shift assays to identify any possible protein-DNA interactions of the RecX protein to the upstream regions of *M. tuberculosis* and *M. smegmatis* *recA*.

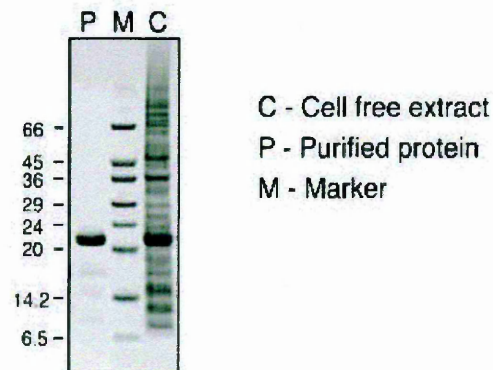
The *M. smegmatis* and *M. tuberculosis* *recA* upstream regions were amplified by PCR. The *M. smegmatis* *recA* upstream region of 609bp including 80bp of the *recA* gene and

promoter, was amplified from DNA template pKP68 (Methods Table 2). The *M. tuberculosis recA* upstream region of 532bp including 93bp of the *recA* gene and its promoter, was amplified from DNA template pKP77 (Methods Table 2).

The DNA was labelled using  $\gamma$ -<sup>32</sup>P ATP and the double-stranded oligonucleotides were diluted. Control binding reactions were carried out with LexA (source: purified within the department by another lab) for each DNA sequence, using 100ng of LexA per reaction. LexA was used as a positive control because it is known to bind the *recA* promoter region. The same method was followed for RecX using 918ng protein, and three subsequent ten-fold dilutions. The samples were then applied to a pre-electrophoresed native 8% (w/v) polyacrylamide gel.

While 100ng LexA resulted in a shift of the labelled DNA in its entirety, no retardation of the probe was detectable even with almost 10 times this amount of RecX (Figure 14). Thus, it appears that RecX does not bind to the *recA* upstream regions of *M. smegmatis* or *M. tuberculosis*. This suggests that RecX does not regulate *M. tuberculosis* or *M. smegmatis* RecA function by binding to the *recA* promoter region to modulate expression by transcriptional control.

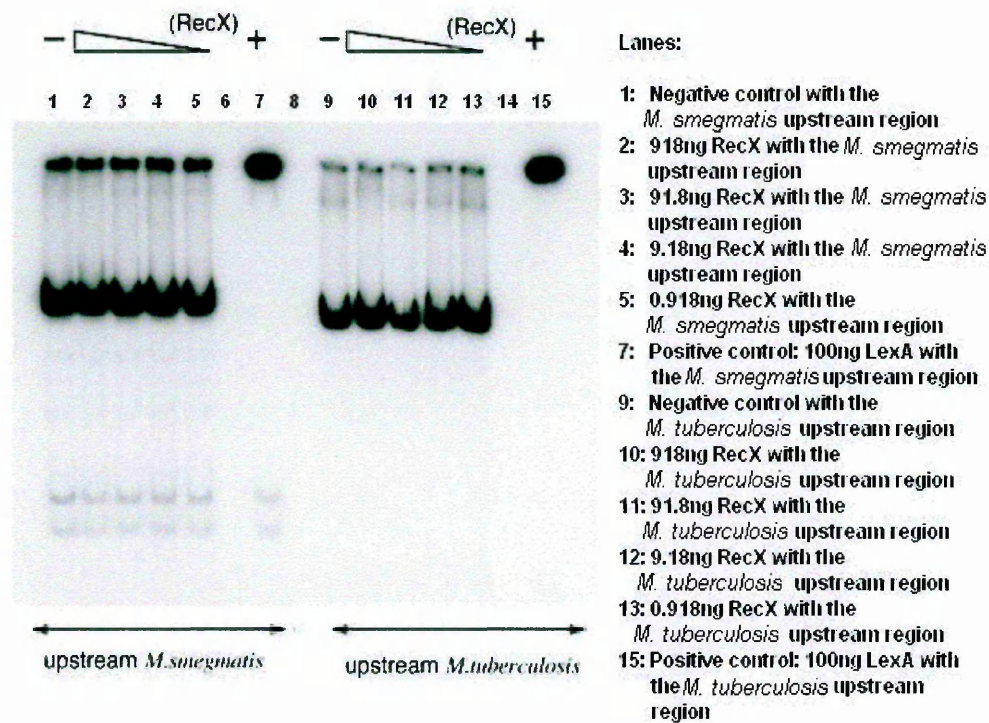
**FIGURE 13 : Purified *M. tuberculosis* RecX protein**



The *M. tuberculosis* *recX* gene was cloned into the pET15b vector. Plasmids were transformed into *E. coli* BL21 (DE3) pLysS cells and the transformants grown and further induced with 1mM IPTG at 37 C for 3 hours. The *M. tuberculosis* RecX protein was found to be present in the pellet and the supernatant. The protein was expressed and then purified by means of a His tag, followed by size exclusion chromatography to purify the 22 kDa protein. The purified *M. tuberculosis* RecX protein was visualised following electrophoresis through a SDS-PAGE (15% polyacrylamide) gel.



**FIGURE 14: DNA mobility shift assay to investigate DNA-protein interactions between RecX and *M. smegmatis* and *M. tuberculosis* promoter regions**



The *recA* upstream regions of *M. smegmatis recA* (609bp including 80bp of the *recA* gene) and *M. tuberculosis recA* (532bp including 93bp of the *recA* gene), were PCR amplified followed by labelling with gamma-32P ATP, and binding with 918ng RecX and three subsequent ten-fold dilutions. LexA was used as a positive control using 100ng of LexA with the *M. smegmatis* and the *M. tuberculosis* upstream regions. The samples were applied to a pre-electrophoresed native 8% (w/v) polyacrylamide gel. The DNA mobility shift assay shows no binding of the RecX protein to the *recA* upstream regions of *M. smegmatis* and *M. tuberculosis*.

### 3.4 SUMMARY

In order to understand the molecular mechanism of a biological process, it is important to identify the protein-protein or protein-DNA interactions which mediate the process. Once these are identified, further experimentation is required to characterise the functional relevance, structure, and regulation of the observed interaction.

A DNA gel shift assay was carried out to investigate if the RecX protein binds to the promoter region of *recA*, thereby modulating RecA activity by transcriptional control. The result of this assay showed no binding to the *recA* upstream regions of *M. smegmatis* and *M. tuberculosis*. A positive result was observed for the positive control LexA protein. This suggests no DNA-protein interaction between *recA* and RecX.

The usefulness of the yeast two-hybrid assay to study mycobacterial protein-protein interactions has been shown previously (Parida *et al.*, 2005; Steyn *et al.*, 2002). In this study, this method was applied to investigate the possibility that RecA and RecX interact *in vivo* by protein-protein interactions. The results of this assay suggest that the two proteins do indeed interact with each other. Positive results were also obtained for the interaction between RecA and RecA, possibly because RecA forms complexes with itself.

However the positive interaction between RecA and RecX was only observed in one orientation and not the other. When expected interacting proteins are *not* recovered they are referred to as 'false negatives'. The reasons for the lack of detection are not always

clear (Vidal and Legrain, 1999), but can include variables such as the level of expression of hybrid proteins, the arrangement of the DNA-binding sites on the reporter genes, the amount of reporter protein required for a detectable phenotype or that the proteins should be able to fold and exist stably in yeast cells and retain their activity as fusion proteins (Phizicky and Fields, 1995). Sometimes proteins may be poorly expressed, or are clipped by yeast proteases. This could result in the predominant population of fusions in the cell being less than full length (Vidal and Legrain, 1999). The lack of interaction observed could be due to any of these reasons.

It is interesting to note that RecA itself tends to bind DNA. However the potential explanation of RecA fused to the activating domain causing activation as is eliminated by the result of the control of PGAD RecA and PGBD vector, which gave a negative result.

The yeast two-hybrid experiments can be taken a step further in order to investigate the exact site of interaction. If interactions are detected by yeast two-hybrid assays, it is possible to make deletions in the genes encoded and identify a minimal domain for interaction. It has been suggested that the likely site of interaction between RecA and RecX is at the highly variable, species-specific C-terminal region of RecA (Karlin & Brocchieri, 1996). This may also be investigated using the yeast two-hybrid system by cloning various partial sequences of RecA and screening for interactions with RecX.

## CHAPTER 4

### RESULTS

#### THE ROLE OF RECX IN MYCOBACTERIA: AN *IN VITRO* ANALYSIS

##### 4.1 AIMS

These sets of experiments are aimed at investigating the hypothesis that RecX acts as a negative modulator of RecA activity. These experiments involved *in vitro* studies of the influence of RecX on two essential biochemical activities of RecA in the cell: ATP hydrolysis and LexA cleavage.

It has been found that *in vitro*, purified RecA mimics strand exchange between two homologous DNA molecules and stimulates autocleavage of the LexA repressor (Kowalczykowski *et al.*, 1994; Roca and Cox, 1990; Ellouze *et al.*, 1999). These activities are dependent on the formation of a nucleoprotein filament in which RecA binds to ssDNA.

RecA activation occurs when ATP is present as a cofactor for RecA to interact with ssDNA. RecA forms a filamentous complex, in which the RecA subunits are organized in



a helical manner around the DNA. In the presence of ATP, the RecA filament is elongated, there is an increase in the size of the helical pitch, a decrease in the diameter and consequently the DNA binding affinity of RecA is increased (Kowalczykowski, 1994; Roca and Cox, 1990; Ellouze *et al.*, 1999). The interaction of RecA with DNA can be monitored by assaying its DNA-dependent ATPase activity.

The first sets of experiments were carried out to study of the influence of RecX on the ATP hydrolysis activity of RecA. This was done by means of the *in vitro* study of this RecA activity, with the subsequent addition of RecX to the reaction and an analysis of the effect of *M. tuberculosis* RecX on the hydrolysis of ATP by *M. tuberculosis* RecA.

The second study involves an *in vitro* analysis of the influence of RecX on the LexA cleavage activity of RecA. When it is activated, RecA plays a role as a co-protease and stimulates the cleavage of the LexA repressor (Little, 1991). The second set of experiments involve the *in vitro* study of this mechanism, with the addition of RecX to the reaction in order to determine the effect of *M. tuberculosis* RecX on the cleavage of *M. tuberculosis* LexA by *M. tuberculosis* RecA.

#### **4.2 *M. TUBERCULOSIS* RECX INHIBITS RECA ATPASE ACTIVITY *IN VITRO*.**

*M. tuberculosis* RecA was activated in a reaction buffer, which also included radioactively labelled [ $\alpha$ -<sup>32</sup> P] dATP and single-stranded DNA. The reaction mix was then divided into aliquots, which were incubated at 37°C. Aliquots were removed at

different time points at which the ATP hydrolysis process was chemically stopped. The samples were analysed by thin layer chromatography and a phosphoimager volume analysis was carried out to obtain quantitative data. Each of the experiments was carried out three times and the mean percentage ATP hydrolysis values for each time point were calculated.

The assay results for the experiments carried out using *M. tuberculosis* RecA showed a clear hydrolysis (reduction) of ATP over time and a clear increase in ADP production over time (See Figure 15 for a typical experiment Table 4 for data and Figure 20 for graph). The assay was carried out three times (see figures 31 and 32 in Appendix for repeat experiments).

The assay was then repeated with the addition of RecX after the activation of RecA, with a RecA: RecX molar ratio of 1:3. The order of addition was: Tris HCl, BSA, MgCl<sub>2</sub>, DTT, RecA, ssDNA storage on ice for RecA activation to occur, followed by addition of RecX and then radioactive ATP to start the reaction. When compared to the previous RecA assay, the results showed a reduction in amounts of ADP produced and therefore in RecA ATPase activity, with the addition of RecX (See Figure 16 for a typical experiment, Table 4 for data and Figure 20 for graph). RecX is therefore acting as a negative modulator to inhibit the ATPase activity of RecA. The assay was carried out three times (see figures 33 and 34 in Appendix for repeat experiments).

As a control, to test for the specificity of the reaction, the assay was carried out with the non-specific protein, calcium binding protein (CBP) from *Entamoeba histolytica*, also added after the activation of RecA. When compared to the results obtained for RecA, it was found that inhibition of RecA ATPase activity did not occur with this non-specific protein (See Figure 17 for a typical experiment, Table 4 for data and Figure 20 for graph). This negative control validates the results obtained for the RecX-RecA ATPase assay. The assay was carried out three times (see figures 35 and 36 in Appendix for repeat experiments).

The assay was repeated with the addition of *M. tuberculosis* RecX at a stoichiometric ratio of 1:1 to RecA (See Figure 18 for a typical experiment, Table 4 for data and Figure 20 for graph), with RecX being added after the activation of RecA. A reduction in ATPase activity was observed when compared to the RecA assay, however the inhibition was not very different from that observed for the 1:3, RecA:RecX assay. The assay was carried out three times (see figures 37 and 38 in Appendix for repeat experiments).

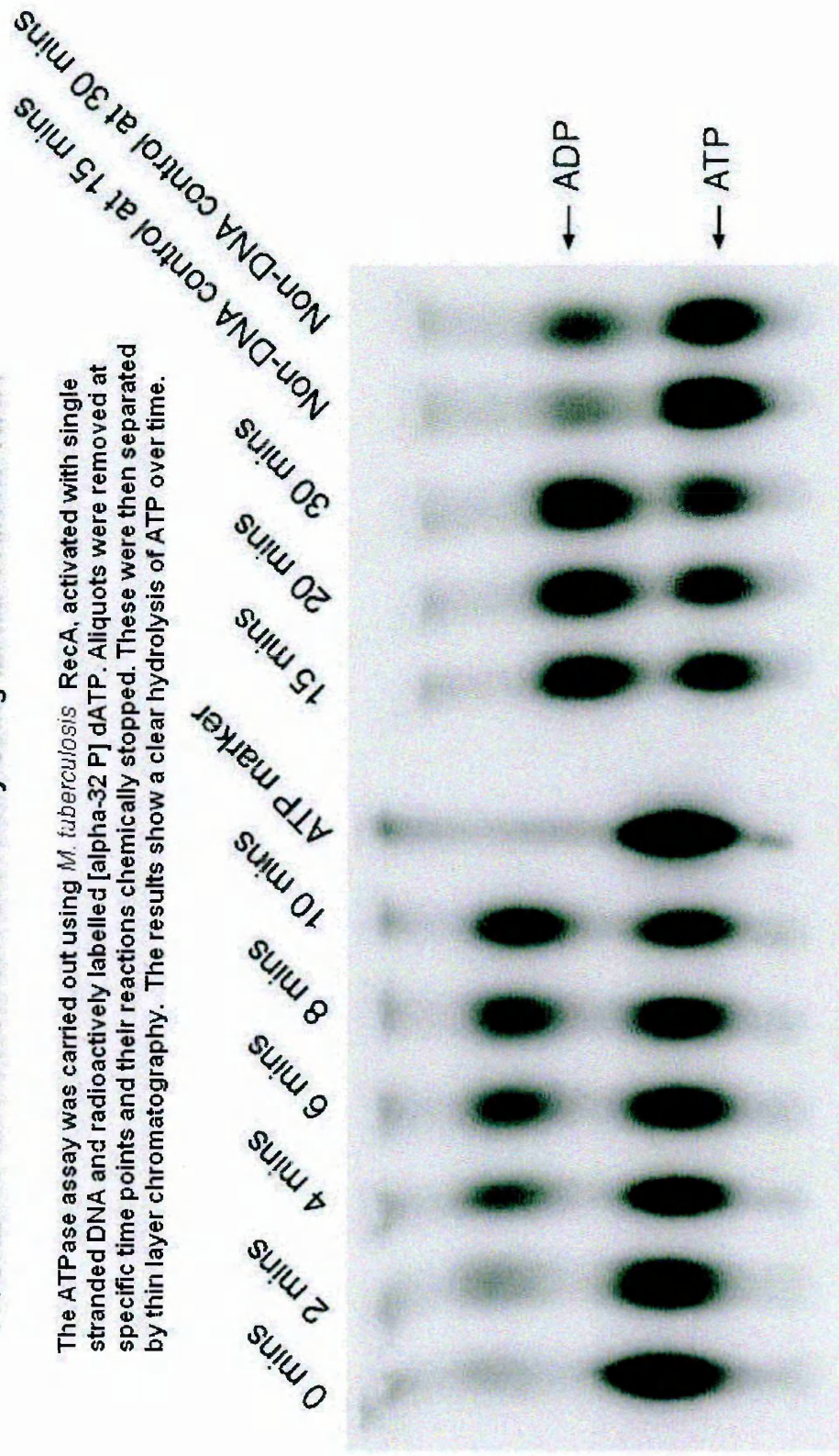
RecA activity requires the formation of a RecA-ssDNA-ATP nucleoprotein filament. Therefore any interference in either the formation or integrity of the nucleoprotein filament will affect RecA activity (Stohl, 2003). To study the mechanism by which RecX negatively modulates RecA ATPase activity, RecX was added to the reaction mix before the activation of RecA by ssDNA at a RecA:RecX ratio of 1:3. ATPase activity results were compared to earlier assays in which RecX was added after RecA activation by ssDNA. It was found that there is a reduced inhibition of ATPase activity when RecX is

added before activation of RecA by ssDNA (See Figure 19 for a typical experiment, Table 4 for data and Figure 20 for graph). Therefore when RecX is not present during the activation stage of RecA, it is less able to affect RecA ATPase activity. The assay was carried out three times (see figures 39 and 40 in Appendix for repeat experiments).

The mean values for the percentage ADP produced over the time course were calculated for the three sets of each of the above experiments and all data sets plotted in the form of a graph for clear comparison (Table 4 and Figure 20).

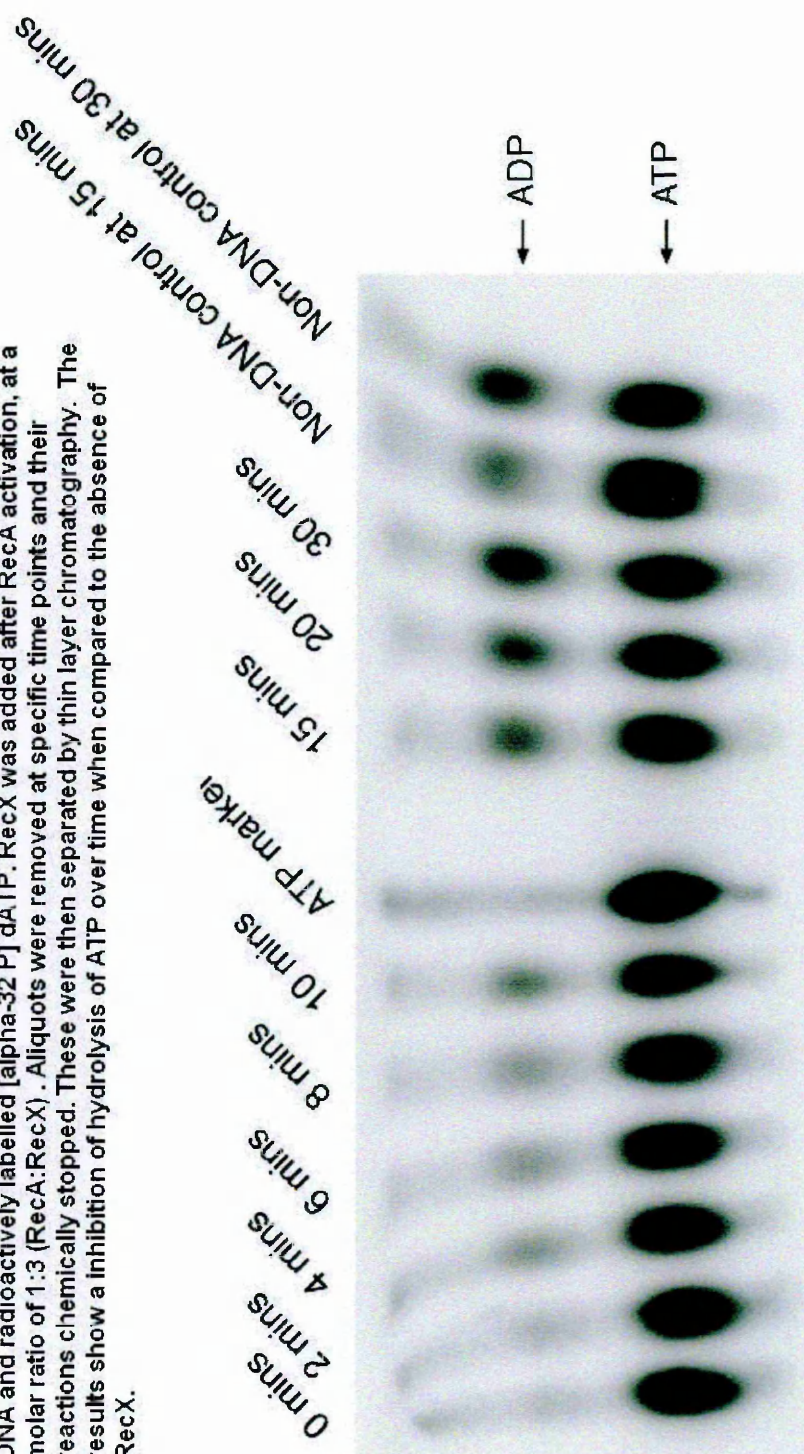
**FIGURE 15: Time Course ATPase Assay Using *M. tuberculosis* RecA**

The ATPase assay was carried out using *M. tuberculosis* RecA, activated with single stranded DNA and radioactively labelled [ $\alpha$ - $^{32}$ P] dATP. Aliquots were removed at specific time points and their reactions chemically stopped. These were then separated by thin layer chromatography. The results show a clear hydrolysis of ATP over time.



**FIGURE 16: Time Course ATPase Assay Using *M.tuberculosis* RecA and RecX**

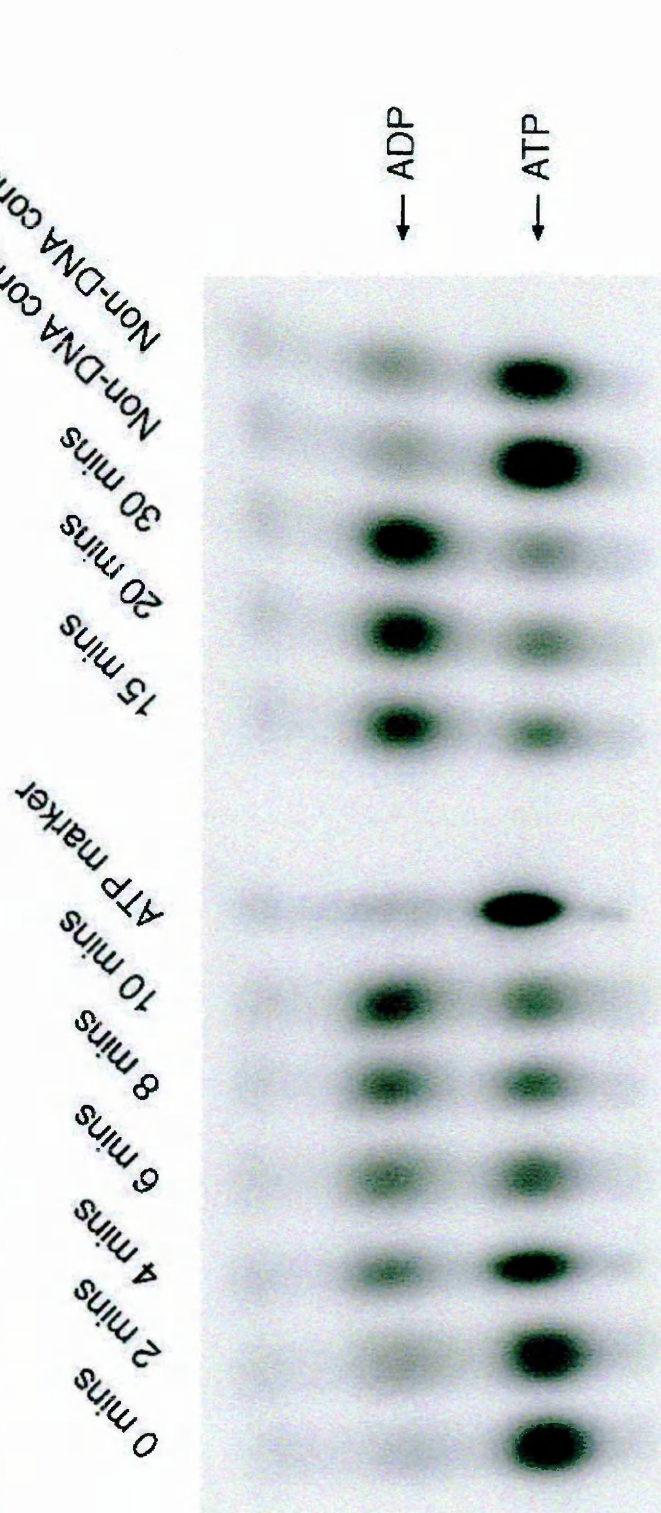
The ATPase assay was carried out using *M. tuberculosis* RecA, activated with single stranded DNA and radioactively labeled [ $\alpha$ - $^{32}$ P] dATP. RecX was added after RecA activation, at a molar ratio of 1:3 (RecA:RecX). Aliquots were removed at specific time points and their reactions chemically stopped. These were then separated by thin layer chromatography. The results show a inhibition of hydrolysis of ATP over time when compared to the absence of RecX.





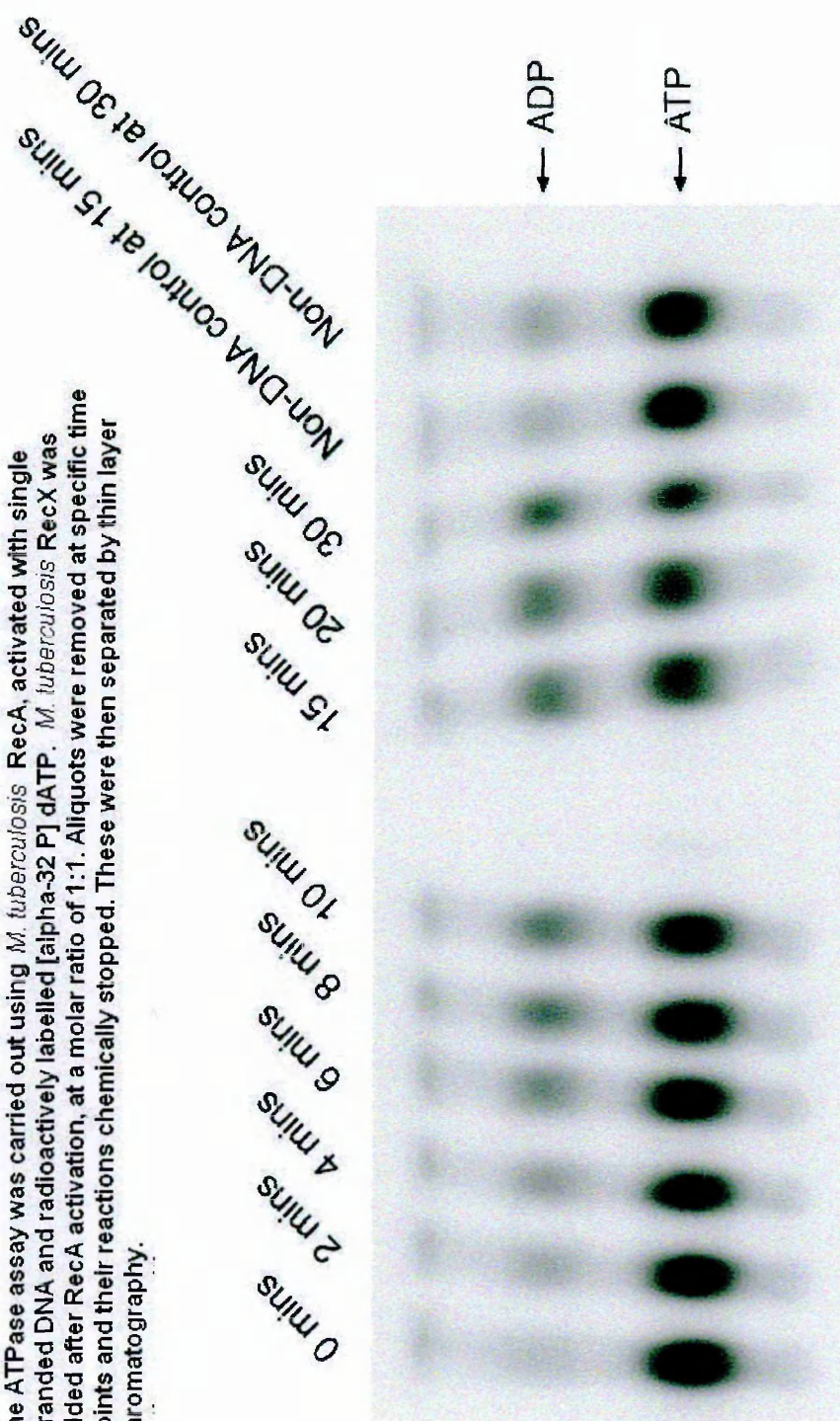
**FIGURE 17 : Time Course ATPase Assay Using *M. tuberculosis* RecA and Calcium Binding Protein**

The ATPase assay was carried out using *M. tuberculosis* RecA, activated with single stranded DNA and radioactively labelled [ $\alpha$ - $^{32}$ P] dATP. The non-specific protein, calcium binding protein (CBP) from *Entamoeba histolytica* was added after RecA activation, at a molar ratio of 1:3. Aliquots were removed at specific time points and their reactions chemically stopped. These were then separated by thin layer chromatography. The results show inhibition of ATPase activity does not occur with this non-specific protein.



**FIGURE 18: Time Course ATPase Assay Using *M.tuberculosis* RecA and RecX in a 1:1 Ratio**

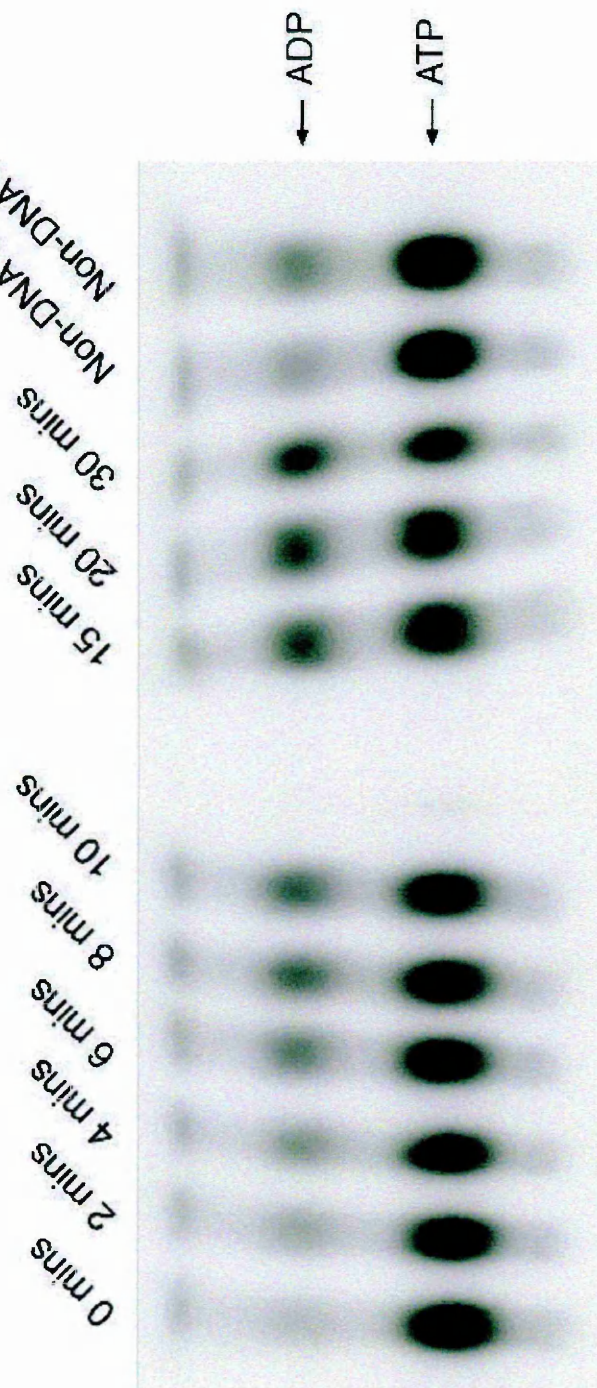
The ATPase assay was carried out using *M. tuberculosis* RecA, activated with single stranded DNA and radioactively labelled [ $\alpha$ - $^{32}$ P] dATP. *M. tuberculosis* RecX was added after RecA activation, at a molar ratio of 1:1. Aliquots were removed at specific time points and their reactions chemically stopped. These were then separated by thin layer chromatography.





**FIGURE 19 : Time Course ATPase Assay Using *M. tuberculosis* RecA and Adding RecX Before RecA Activation**

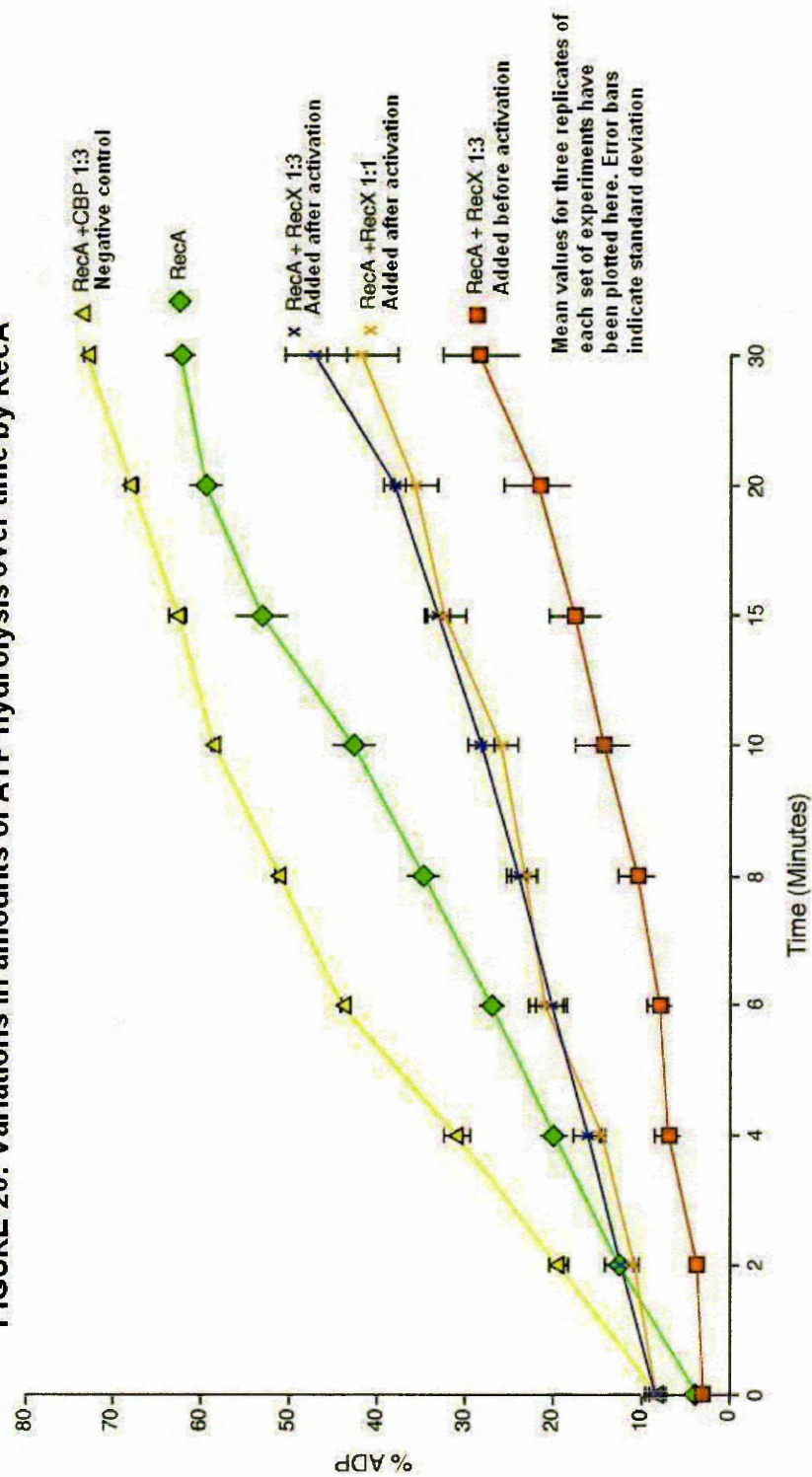
The ATPase assay was carried out using *M. tuberculosis* RecA, activated with single stranded DNA and radioactively labelled [ $\alpha$ - $^{32}$ P] dATP. *M. tuberculosis* RecX was added to the reaction mix before activation of RecA and at a RecA:RecX ratio of 1:3. Aliquots were removed at specific time points and their reactions chemically stopped. These were then separated by thin layer chromatography.



**Table 4: Mean values for %ADP over time for three sets of each experiment**

| <b>Time<br/>Minutes</b> | <b>RecA</b> | <b>RecA+<br/>RecX</b> | <b>RecA+<br/>CBP</b> | <b>RecA+<br/>RecX 1:1</b> | <b>RecA + RecX<br/>Before Activation</b> |
|-------------------------|-------------|-----------------------|----------------------|---------------------------|--|
| 0                       | 3.54        | 2.98                  | 8.16                 | 8.29                      | 8.57                                     |
| 2                       | 12.64       | 3.68                  | 19.4                 | 10.81                     | 12.46                                    |
| 4                       | 19.95       | 6.99                  | 30.86                | 14.45                     | 16.08                                    |
| 6                       | 26.91       | 7.97                  | 43.81                | 20.73                     | 20.16                                    |
| 8                       | 34.66       | 10.47                 | 50.84                | 23.25                     | 24.02                                    |
| 10                      | 42.49       | 14.32                 | 58.41                | 25.97                     | 28.04                                    |
| 15                      | 53          | 17.47                 | 62.57                | 32.16                     | 32.92                                    |
| 20                      | 59.32       | 21.77                 | 67.74                | 35.52                     | 37.84                                    |
| 30                      | 62.28       | 28.12                 | 72.81                | 41.5                      | 46.79                                    |

**FIGURE 20: Variations in amounts of ATP hydrolysis over time by RecA**



#### **4.3 *M. TUBERCULOSIS* RECX INHIBITS THE LEXA CLEAVAGE ACTIVITY OF RECA *IN VITRO*.**

RecA co-protease activity stimulates the autocatalytic cleavage of LexA. For this assay, RecA was first activated with single-stranded DNA and ATP $\gamma$ S. In addition to ATP, RecA activity can be promoted by deoxyadenosine triphosphate (dATP), which is a nucleotide precursor used in cells for DNA synthesis, and adenosine 5'-O-(3-thio)triphosphate (ATP $\gamma$ S), which is a hydrolysis-resistant adenosine triphosphate (ATP) analog. Following activation, the samples were then incubated with LexA at 37°C for 1 hour to allow LexA cleavage to occur. The samples were separated by acrylamide gel electrophoresis, stained and analysed. The cleaved products can be observed as individual bands in the gel (**Figure 21**).

Reactions were prepared with RecX at different concentrations, ranging from RecA: RecX molar ratios of 1:0.2 up to 1:3. A gradual increase in the inhibition of LexA cleavage was observed (**Figure 21**).

In order to test for the specificity of this reaction, control experiments were carried out with bovine serum albumin (BSA) (obtained commercially), NusB (a transcription antitermination protein purified within the division) from *M. tuberculosis*, S10 (a small ribosomal subunit protein purified within the division) from *M. tuberculosis* and calcium binding protein (CBP) (commercial) from *Entamoeba histolytica*. Inhibition of LexA cleavage did not occur with these non-specific proteins (**Figures 22 and 23**).

RecA activity requires the formation of a RecA-ssDNA-ATP nucleoprotein filament. Therefore any interference in either the formation or integrity of the nucleoprotein

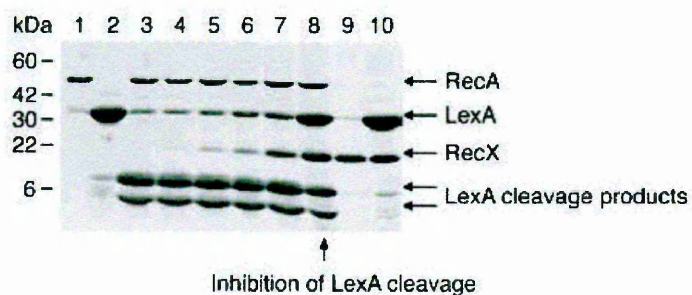
filament will affect RecA activity (Stohl *et al.*, 2003). There are several possible mechanisms through which RecX may be able to inhibit RecA co protease activity. In this *in vitro* model, it is possible that RecX caused inhibition of LexA cleavage by sequestering the single-stranded DNA present in the reaction mix. This would negatively influence the activation state of RecA and inhibit LexA cleavage.

In an attempt to understand the mechanism through which RecX inhibits RecA co-protease activity, RecX protein was added to the reaction mix either before or after the activation of RecA and the level of inhibition compared. The inhibition of LexA cleavage was observed to be the same in both cases (**Figure 23**).

The DNA concentration in the reaction mix was increased to 5x and 10x the original concentration. It was observed that increasing the DNA concentration did not influence the LexA cleavage activity. The intensity and size of the cleaved LexA products were identical to those observed for the original DNA concentration (**Figure 24**). This result strongly suggests that RecX interacts directly with RecA, to inhibit its co protease activity and not through the sequestering of ssDNA.

At 37°C and pH 10, LexA undergoes cleavage, without the influence of RecA pH-dependent auto-cleavage of LexA was investigated by incubating LexA in a 0.5M CAPS buffer at pH10 containing 0.5M NaCl at 37°C for 24 hours. RecX was included in this mix and the effect on LexA cleavage assessed. Incubations at 4°C served as controls. RecX was found to have no inhibitory or enhancing effect on LexA autocleavage activity, suggesting that RecX does not interact directly with LexA to inhibit LexA cleavage (**Figure 25**).

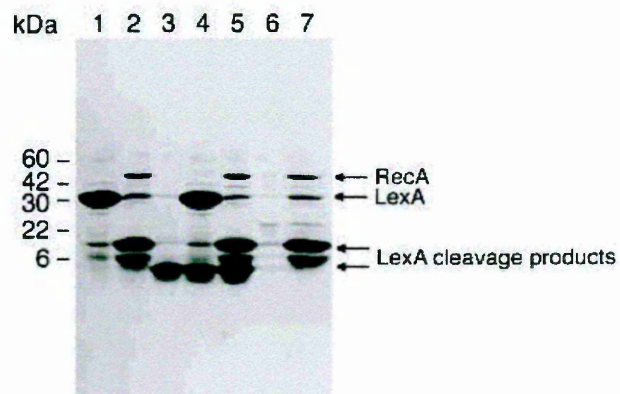
**FIGURE 21: RecA Mediated LexA Cleavage Assay  
Showing the Inhibition of Cleavage by RecX**



1. RecA
2. LexA
3. RecA + LexA
4. LexA + RecA + RecX 1:0.2
5. LexA + RecA + RecX 1:0.5
6. LexA + RecA + RecX 1:1
7. LexA + RecA + RecX 1:2
8. LexA + RecA + RecX 1:3
9. RecX
10. LexA + RecX

A RecA-mediated LexA cleavage assay showing the inhibition of the LexA cleavage reaction with the presence of 0.2 to 3x as much RecX as RecA in the reaction mix. Proteins were separated through a 15% SDS PAGE gel.

**FIGURE 22: RecA Mediated LexA Cleavage Assay  
Showing No Inhibition of Cleavage by Non-Specific Proteins (Calcium binding protein and NusB)**



1. LexA
2. RecA + LexA
3. Calcium Binding Protein (CBP)
4. CBP + LexA
5. CBP + LexA + RecA
6. NusB
7. NusB + LexA + RecA

A RecA-mediated LexA cleavage assay showing the effects of including the non-specific proteins Calcium binding protein and NusB in place of RecX in a 1:3 ratio in relation to RecA. Proteins were separated through a 15% SDS-PAGE gel.



**FIGURE 23: RecA Mediated LexA Cleavage Assay  
Showing Effect of Non-Specific Proteins (S10, NusB,  
BSA) and Addition of RecX Before and After  
Activation of RecA**



1. RecA
2. RecX
3. LexA
4. RecX + LexA
5. RecA + LexA
6. RecA + RecX + LexA (before activation)
7. RecA + RecX + LexA (after activation)
8. BSA
9. RecA + BSA + LexA
10. S10
11. RecA + S10 + LexA
12. NusB
13. RecA + NusB + LexA

A RecA-mediated LexA cleavage assay showing the effects of including the non-specific proteins BSA, S10 and NusB instead of RecX in a 1: 3 ratio in relation to RecA. The gel also shows the effects of adding RecX before or after the activation of RecA. Proteins were separated through a 15% SDS-PAGE gel.



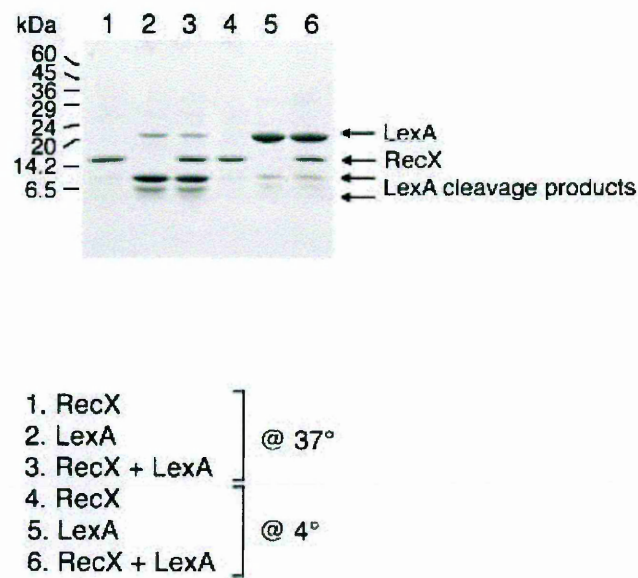
**FIGURE 24: RecA Mediated LexA Cleavage Assay with Varying Amounts of DNA**



1. LexA
2. RecA + LexA (1x DNA)
3. RecA + LexA (5 x DNA)
4. RecA + LexA (10 x DNA)
5. RecA + LexA + RecX (1x DNA)
6. RecA + LexA + RecX (5 x DNA)
7. RecA + LexA + RecX (10 x DNA)

A RecA-mediated LexA cleavage assay showing the effects of adding more ssDNA in the presence of RecX. Proteins were separated through a 15% SDS-PAGE gel.

**FIGURE 25: pH Dependent LexA Cleavage Assay with the Addition of RecX**



A pH-dependent LexA cleavage assay showing that RecX does not have an affect on this reaction. A negative control was carried out at 4C, at which the LexA cleavage does not occur. Proteins were separated through a 15% SDS-PAGE gel.

#### 4.4 SUMMARY

RecA is key in the induction of the SOS pathway of DNA repair and mutagenesis to maintain the integrity of the genome (Drees *et al.*, 2004b). RecA functions as a nucleoprotein filament, with filaments assembling on single-stranded DNA (ssDNA), assembling and disassembling in the 5' to 3' direction. The protomers are added to one end and subtracted from the other end under the appropriate conditions for this reaction (Drees *et al.*, 2004b). The rate of extension of RecA filaments during assembly is faster than the end-dependent disassembly, thereby leading to the formation of long filaments (Lusetti *et al.*, 2004). In this form, RecA acts as a DNA-dependent ATPase (Drees *et al.*, 2004b).

In the form of a RecA-ssDNA-ATP nucleoprotein filament, RecA acts as a recombinase and promotes various DNA pairing and strand exchange reactions between single-stranded DNA and another homologous DNA molecule (Stohl *et al.*, 2003). Electron microscopy and X-ray crystallography studies have shown that ATP binds at a subunit interface, leading to ATP mediated conformational changes within RecA, allowing the protein to catalyze DNA strand-exchange (Bell, 2005).

The RecA protein also regulates induction of the SOS response as a co protease by facilitating the autocatalytic cleavage of the LexA repressor (Kim and Little 1993; Nastri and Knight 1994; Drees *et al.*, 2004a). In *E.coli*, the LexA protein represses the transcription of over 30 SOS genes of the SOS regulon (Stohl *et al.*, 2003). Upon DNA damage and the development of ssDNA breaks, RecA forms nucleoprotein filaments, described above, which then act as a co protease to enhance the expression of the SOS genes through the autocatalytic cleavage of LexA.

The hypothesis that RecX acts by down regulating RecA activity was explored in this study by means of two *in vitro* assays, the ATPase assay and the LexA cleavage assay, to study these two essential activities of RecA and the influence of RecX on them. It was observed from these assays that RecX has an inhibitory effect on both the ATPase and LexA cleavage activities of RecA. Non-specific proteins were tested as negative controls in place of RecX and no inhibitory activity was observed for these. Therefore the inhibition observed was specific to RecX.

When the concentration of RecX was varied, it was found in the LexA cleavage assay that an increased concentration of RecX, tested up to a RecA:RecX ratio of 1: 3, results in increased levels of inhibition of both activities of RecA. Therefore RecX inhibition of LexA cleavage *in vitro* is concentration dependent.

In order to investigate the mechanism by which RecX is able to negatively modulate RecA activity, in both assays, RecX was added to the reaction before and after RecA activation with ssDNA. It was found for the ATPase assays that there is a higher level of inhibition when RecX is added to the reaction before RecA activation. No obvious difference was observed for the LexA cleavage assay. However it possible that there may be a difference which cannot be seen by eye on the PAGE gel, but might be observed if the bands were quantified by volumetric analysis.

There could be many possible ways in which RecX interferes with RecA activity more effectively if added before RecA is activated. RecX may be interfering with the formation or integrity of the RecA-ssDNA-ATP complex by interacting directly with the ssDNA or ATP, making them unavailable to RecA for activation, or by interacting directly with the RecA, and changing its conformation to block the formation of the nucleoprotein filaments.

It was found that when the amount of ssDNA was increased to 5x and 10x the original concentration, for the LexA cleavage assays, that this did not change the level of inhibition of LexA cleavage caused by RecX. Therefore it is unlikely that RecX inhibits RecA activity by sequestering or directly interacting with the ssDNA to make it unavailable to RecA for activation.

Another possibility for the mechanism of RecX inhibition of LexA cleavage could be by the direct interaction of RecX with the LexA protein, thus affecting LexA cleavage by

RecA. To investigate this possibility, RecX was included in a pH dependent LexA cleavage assay in which LexA is cleaved independent of RecA. The addition of RecX to this reaction had no effect on LexA cleavage activity. This suggests that RecX does not interact directly with LexA.

Since this work was carried out, a similar result has been confirmed in the case of *E. coli*, in which RecA co-protease and ATPase activities have also been shown to be inhibited by RecX (Stohl *et al.*, 2003; Galvao *et al.*, 2004). One mechanism proposed suggests that RecX causes inhibition through the disassembly of activated RecA filaments. When RecX protein triggers RecA filament disassembly, the fragments are still active as the disassembly proceeds, causing LexA cleavage, and ATP hydrolysis to decline at a gradual pace.

Evidence has also been found to suggest that RecX binds along the length of a RecA filament when high levels of RecX are present (Vanloock *et al.*, 2003; Drees *et al.*, 2004a). Thus a possible reason for the requirement of a high ratio of RecX compared to RecA for clear inhibition in this study could be because all monomers in the RecA nucleoprotein filament hydrolyze ATP and contribute to net ATPase activity. Therefore, RecX is not just binding to a localized region and higher concentrations of RecX would thus be required relative to RecA (Venkatesh *et al.*, 2002).

In conclusion, it was found from this study that RecX acts as a negative modulator of RecA ATPase and LexA cleavage activities. This inhibition is specific and concentration

dependent. RecX interferes with the activation stage of RecA to have this effect. Since this work was carried out, there have been several publications supporting these results and describing in further detail the mechanism by which RecX is able to negatively modulate these activities of RecA.

## CHAPTER 5

### RESULTS

#### THE ROLE OF RECX IN MYCOBACTERIA:

#### AN *IN VIVO* ANALYSIS

##### 5.1 AIMS

These sets of experiments are aimed at characterizing the consequences *in vivo* of the effect of RecX on RecA biochemical activity. RecA has a key role in homologous recombination and is involved in the DNA damage response within the cell, both playing a direct role in repair and controlling the expression of other DNA repair genes via the inactivation of LexA. The effect of RecX on these activities of RecA was studied by assaying recombination frequencies, survival following DNA damage, and RecA levels following DNA damage.

To carry out these assays, differential complementation of a *recA-recX* deletion mutant of *M. smegmatis* termed HS42 (Papavinasandaram *et al.*, 1998) was exploited. A prior study had shown that complementation of this strain with plasmid pKP108, which contains *M.*



*smegmatis* *recA* and *recX* expressed from the native *recA* promoters in the vector pMV306, restored both UV resistance and recombination frequencies to wild-type levels (Papavinasundaram *et al.*, 1998). Complementation with *recA* alone in an otherwise identical construct (pKP107) appeared to be less effective in restoring recombination frequencies although no difference in UV resistance was observed. pMV306 integrates at a L5 mycobacteriophage attachment site and it permits the analysis of complementation by genes present in single copy. This is a distinct advantage over experiments based on multicopy plasmids.

In this investigation, additional data for recombination frequencies following complementation with these two plasmids was obtained to clarify if the presence of *recX* made a significant difference. In addition, a potential role of *recX* in the other properties indicated above was assessed by comparing HS42 carrying each of the two plasmids.

These strains were applied to a homologous recombination assay to determine the role of *recX* in recombination. The second assay was a cell viability assay to determine the role of *recX* in cell survival following DNA damage treatment by mitomycin C. This was carried out to further investigate the potential role of *recX* in survival following DNA damage. Survival following exposure to another damaging agent, mitomycin C, was therefore assessed. The third assay was a time-course assay to study the role of *recX* on RecA recovery levels following pulse DNA damage treatment.

## **5.2 PHENOTYPE OF THE *RECA-RECX* COMPLEMENTED STRAINS.**

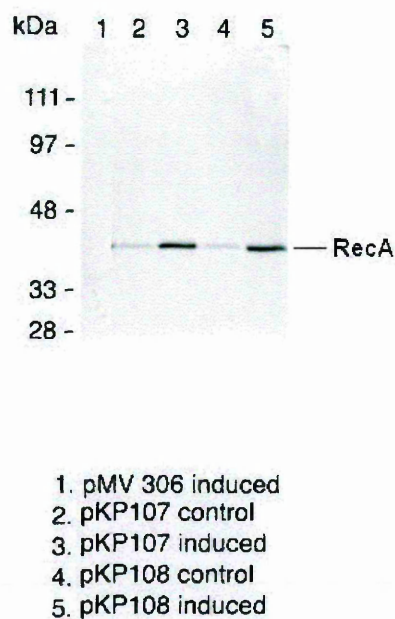
In order to verify the expression of RecA in the HS42 strain complemented with plasmids pKP107 (*M. smegmatis recA*) and pKP108 (*M. smegmatis recA+recX*), cell free extracts were produced from cultures induced with mitomycin C, which causes DNA damage and therefore raises levels of RecA production, as well as from untreated cultures. A Western assay was carried out using antibodies against RecA. The Western blot clearly showed the presence of RecA in the strains carrying the clones but not that transformed with the vector control, pMV306 (Figure 26). Furthermore, upon induction of DNA damage by mitomycin C the RecA expression level was increased as expected. RecX does not seem to have an effect on wild type levels of RecA and no difference was seen in induction of RecA between pKP107 and pKP108 after 5 hours.

## **5.3 RECX INCREASES THE FREQUENCY OF HOMOLOGOUS RECOMBINATION.**

In all organisms, homologous recombination is an essential function, central to genetic exchange, maintenance of chromosomal integrity, DNA repair and DNA replication. RecA is a key element in these processes. An investigation was carried out to determine whether RecX influences RecA recombination activity. The *M. smegmatis* deletion mutant complemented with the *M. smegmatis recA* and *recA+recX* constructs described above was used in a homologous recombination assay to study numbers of double crossover events in the various complemented strains.

The assay involved the use of *pyrF* and fluoroorotic acid. Generally, Fluoroorotic acid (5-fluorouracil-6-carboxylic acid monohydrate) is used to detect expression of the *pyrF* gene, which encodes orotidine-5'-phosphate decarboxylase. Cells with an active *pyrF* gene convert the 5-FOA to fluorodeoxyuridine, which is toxic. Cells carrying a mutation in the *pyrF* gene grow in the presence of FOA, if the medium is supplemented with uracil.

**FIGURE 26: Western Analysis of RecA Production**



Western blot showing the expression of RecA in the *M. smegmatis* HS42 deletion-mutant cells complemented with *M. smegmatis recA*, *M. smegmatis recA+recX*. RecA was induced by treatment of cultures with mitomycinC. Uninduced cultures produced constitutive expression of RecA, while the induced cultures produced a significantly higher level of the protein.

This assay essentially involved the *pyrF* gene being disrupted by a gentamycin resistance cassette, in a non-replicating vector, which was then introduced into *M. smegmatis* cells. If the resistance gene integrates into the chromosome then gentamycin resistant colonies could arise by three mechanisms: double crossovers, single crossovers and random integration. Double crossovers involve the replacement of the wild type *pyrF* gene with the disrupted allele. These can be identified because the mutated *pyrF* allele gives resistance to FOA and also uracil auxotrophy. The single crossovers and random integrations result in integration of the whole plasmid. In the case of the latter two, both the wild type and mutated alleles are present.

Five sets of data were obtained for separate experiments and their mean values were calculated (**Tables 5 and 6**). It was found that the ratio of double crossover events to the sum of single crossover events and random integrations for *recA+recX* is higher at a value of 0.26 compared to *recA* strains at 0.17.

**Table 5: CFU Data obtained for Recombination Assay.**

|  | CFUs on<br>7H11+<br>Gentamicin+<br>Uracil     | CFUs on<br>7H11+<br>FOA  |  |
|--|---|--------------------------|--|
| Strain   | Single<br>crossovers+<br>Random<br>integrants | Double<br>Cross<br>overs | Ratio of Double crossovers:<br>Single Crossover+Random<br>integrants |
| <i>M. smegmatis</i> <i>recA-recX</i><br>mutant complemented with<br><i>recA</i> ,<br>pKP 107:      |   |                          |  |
| Experiment 1   | 109   | 17                       | 0.156  |
| Experiment 2   | 128   | 17                       | 0.133  |
| Experiment 3   | 18  | 4                        | 0.222  |
| Experiment 4   | 21  | 5                        | 0.238  |
| Experiment 5   | 110   | 11                       | 0.10   |
| <i>M. smegmatis</i> <i>recA-recX</i><br>mutant complemented with<br><i>recA+recX</i> ,<br>pKP 108: |   |                          |  |
| Experiment 1   | 104   | 21                       | 0.202  |
| Experiment 2   | 102   | 21                       | 0.206  |
| Experiment 3   | 54  | 21                       | 0.389  |
| Experiment 4   | 58  | 18                       | 0.31   |
| Experiment 5   | 102   | 20                       | 0.197  |

**Table 6: Mean values for homologous recombination assay results, calculated from five sets of experiments.**

| Strain   | Expt 1<br>Ratio<br>Double<br>Cross<br>Overs | Expt 2<br>Ratio<br>Double<br>Cross<br>overs | Expt 3<br>Ratio<br>Double<br>Cross<br>overs | Expt 4<br>Ratio<br>Double<br>Cross<br>overs | Expt 5<br>Ratio<br>Double<br>Cross<br>overs | Mean<br>Ratio<br>Double<br>Crossovers |
|--|---|---|---|---|---|---------------------------------------|
| <i>M. smegmatis</i><br><i>recA-recX</i> mutant<br>complemented<br>with <i>recA</i> ,<br>pKP 107      | 0.156                                       | 0.133                                       | 0.222                                       | 0.238                                       | 0.10  | 0.17                                  |
| <i>M. smegmatis</i><br><i>recA-recX</i> mutant<br>complemented<br>with <i>recA+recX</i> ,<br>pKP 108 | 0.202                                       | 0.206                                       | 0.389                                       | 0.31  | 0.197                                       | 0.261                                 |

The two way factorial ANOVA (Analysis of variation) test was carried out for the above data to determine if the values for pKP107 and pKP 108 for each experiment are significantly different. The analysis was carried out using an online ANOVA tool (<http://faculty.vassar.edu/lowry/VassarStats.html>).

The test showed a significant difference between pKP107 and pKP108 values for the double crossover ratio. Therefore

#### 5.4 RECX INCREASES CELL SURVIVAL UPON DNA DAMAGE.

An *in vivo* assay was carried out to determine if RecX promotes survival when expression of *recA* is induced by DNA damage using HS42 complemented with pKP107 and pKP108. The strains were grown in liquid culture till OD<sub>600</sub> of 0.6 and induced with mitomycin C, which causes DNA damage. Induced and uninduced flasks were further incubated and 3 aliquots were taken from each flask and serially diluted from 10<sup>-1</sup> to 10<sup>-6</sup> and plated. Colony numbers were counted and the percentage survival of the different strains compared. Replicates were done for each strain.

It was found that the percentage of cell survival is higher for *M. smegmatis recA+recX*, at 13% compared to  $\Delta recX$ , at 4.3% (**Figure 27, Table 7**).

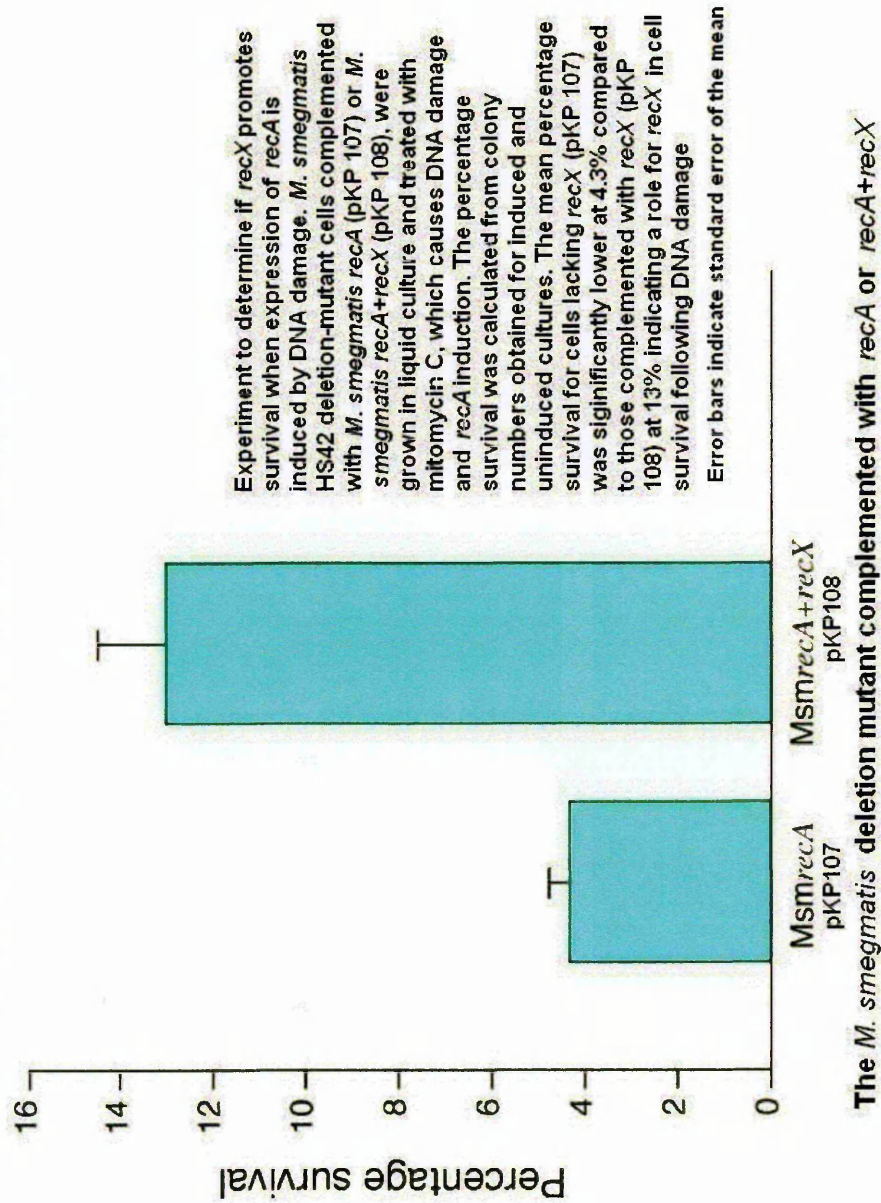


**Table 7: Survival Experiments**

| Strain   | Aliquot   | Induced<br>cfu<br>numbers |      |     | Control<br>cfu<br>numbers |       |      | %<br>survival | Mean<br>%<br>survival |       |
|--|-----------|---------------------------|------|-----|---------------------------|-------|------|---------------|-----------------------|-------|
| Dilution   |           | -3                        | -4   | -5  | -3                        | -4    | -5   |               |                       |       |
| <i>M. smegmatis</i> <i>recA-recX</i><br>mutant complemented with<br><i>recA</i> , pKP 107<br><b>a</b>      | Aliquot 1 | 41                        | 2    | 0   | 600+                      | 52    | 8    | 5.30%         | 4.30%                 |       |
|  | Aliquot 2 | 26                        | 1    | 0   | 600+                      | 113   | 3    |               |                       |       |
|  | Aliquot 3 | 58                        | 1    | 0   | 600+                      | 71    | 6    |               |                       |       |
|  | <b>b</b>  | Aliquot 1                 | 42   | 0   | 0                         | 600+  | 82   | 7             |                       | 3.59% |
| Aliquot 2  |           | 21                        | 1    | 0   | TMTC                      | 92    | 8    |               |                       |       |
| Aliquot 3  |           | 29                        | 3    | 1   | 800+                      | 82    | 8    |               |                       |       |
| <b>c</b>   | Aliquot 1 | 37                        | 6    | 0   | TMTC                      | 133   | 33   | 3.88%         |                       |       |
|  | Aliquot 2 | 58                        | 7    | 0   | TMTC                      | 154   | 54   |               |                       |       |
|  | Aliquot 3 | 56                        | 8    | 0   | TMTC                      | 102   | 10   |               |                       |       |
| <i>M. smegmatis</i> <i>recA-recX</i><br>mutant complemented with<br><i>recA+recX</i> , pKP 108<br><b>a</b> | Aliquot 1 | 600+                      | 211  | 26  | TMTC                      | 1000+ | 142  | 15.35%        |                       | 13%   |
|  | Aliquot 2 | 800+                      | 187  | 18  | TMTC                      | 800+  | 126  |               |                       |       |
|  | Aliquot 3 | 800+                      | 190  | 24  | TMTC                      | 1000+ | 145  |               |                       |       |
|  | <b>b</b>  | Aliquot 1                 | TMTC | 212 | 16                        | TMTC  | 800+ | 127           | 15.78%                |       |
| Aliquot 2  |           | TMTC                      | 224  | 19  | TMTC                      | 800+  | 140  |               |                       |       |
| Aliquot 3  |           | 800+                      | 189  | 7   | TMTC                      | 800+  | 129  |               |                       |       |
| <b>c</b>   | Aliquot 1 | 134                       | 10   | 2   | TMTC                      | 121   | 8    | 11.47%        |                       |       |
|  | Aliquot 2 | 116                       | 18   | 0   | TMTC                      | 98    | 9    |               |                       |       |
|  | Aliquot 3 | 110                       | 8    | 0   | TMTC                      | 95    | 23   |               |                       |       |
| <b>d</b>   | Aliquot 1 | 165                       | 25   | 2   | TMTC                      | 223   | 39   | 9.57%         |                       |       |
|  | Aliquot 2 | 174                       | 17   | 2   | TMTC                      | 248   | 23   |               |                       |       |
|  | Aliquot 3 | 133                       | 23   | 3   | TMTC                      | 146   | 13   |               |                       |       |

To test if the values for pKP 107 are significantly different from those of pKP 108, a standard deviation value was calculated for the % survival values and found to be 4.8. The critical value for a 1% significance test was taken as 2.2. As the standard deviation value lies outside of the critical value, the null can be rejected. Therefore it can be concluded that there is a significant difference between the % survival values obtained for the *recA-recX* mutant complemented with *recA* and the *recA-recX* mutant complemented with *recA+recX*.

**FIGURE 27: Percentage survival following DNA damage for a *M. smegmatis* *recA-recX* mutant complemented with *recA* or *recA+recX***



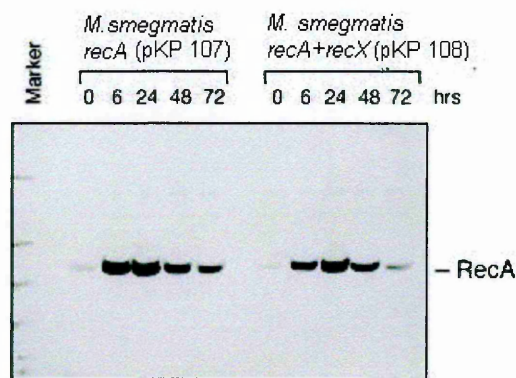
### **5.5 RECX DELAYS THE INDUCTION AND ENHANCES THE RECOVERY OF RECA LEVELS FOLLOWING PULSE DNA DAMAGE.**

DNA damage by Mitomycin C leads to induction of the SOS response and expression of genes involved in this response, including *recA*. Following repair, RecA levels return to normal, basal levels, otherwise this could be deleterious to the cell. This experiment is aimed at studying the effect of *recX* on the induction and recovery of RecA levels in strains of *M. smegmatis*, with and without *recX*, following pulse DNA damage by exposure to mitomycin C.

The strains were treated with a pulse of mitomycin C for one hour in liquid culture and cell free extracts harvested at 0, 6, 24, 48 and 72 hours. RecA levels were analysed by western blots using RecA antibodies.

It was found that RecA peak levels were reached at around 24 hours in both the presence and absence of *recX*. However in the presence of *recX*, the decline to original RecA levels was much more rapid. This suggests that in *M. smegmatis*, *recX* plays a role in enhancing the recovery of RecA to original levels following pulse DNA damage (**Figure 28**) The entire experiment was carried out three times (see figures 41 and 42 in Appendix for repeat experiments).

**FIGURE 28: Western analysis showing variation in RecA levels following pulse DNA damage for *M. smegmatis* *recA-recX* deletion strains complemented with *recA* or *recA+recX***



A *M. smegmatis* *recA-recX* deletion mutant complemented with *M. smegmatis* *recA* (pKP107) and *recA+recX* (pKP108) constructs was treated with a pulse of mitomycin C for one hour. The cultures were then washed with Dubos medium and incubated in medium without mitomycin C for a period of 72 hours. Regular harvests were prepared at 0, 6, 24, 48 and 72 hours. The cell free extracts were prepared and RecA levels were analysed by western blots using RecA antibodies. The experiment was repeated 3 times and the figure shows a typical blot.

## 5.6 SUMMARY

These *in vivo* experiments were carried out to investigate the consequences of RecX modulating the biochemical activities of RecA. The first step was to create strains of a *M. smegmatis* *recA* deletion mutant, complemented with *M. smegmatis* *recA* and *recA+recX*. Expression of RecA was verified by a Western blot on cell free extracts of cultures of the strains. The complemented strains were found to express RecA and the level increased upon DNA damage. RT-PCR experiments were also carried out to verify RecX expression, however these did not give any clear results. Attempts were made to produce RecX antibodies (by another member of the division) to test for RecX expression by Western blots, however this too was unsuccessful.

The results from the viability experiments showed a higher percentage survival for *M. smegmatis* *recA+recX*, at 13% compared to  $\Delta recX$ , at 4.3%. Since it has previously been shown that *recA* overexpression is lethal in a *recX* mutant (Papavinasundaram *et al.*, 1997; Papavinasundaram *et al.*, 1998), an impaired viability of the *recX* mutant after DNA damaging treatment would be expected.

It is possible that the  $\Delta recX$  strains are not inhibited directly by mitomycin C treatment, but due to the toxic action of RecA. It has been shown that an enhanced affinity for DNA by RecA could be responsible for such toxicity (Vierling *et al.*, 2000).

It is possible that there was no significant difference in survival observed following exposure to UV (Papavinsasundaram *et al.*, 1998) because UV sensitivity is a complex phenotype, as indicated by studies carried out in other organisms to test the effect of *recX* on UV sensitivity (Lusetti *et al.*, 2006). In most cases the effects of *recX* mutations on UV sensitivity have been minor. This could also be due to the fact that other SOS-induced genes are also involved in protecting the cell from the effects of RecA (Pages *et al.*, 2003; Stohl *et al.*, 2003, Lusetti *et al.*, 2006, Vierling *et al.*, 2000).

The *in vivo* results for the assay to assess cell survival after exposure to DNA damaging agents support the essential role of the RecA protein in cell survival in the presence of mutagenic agents. The results also indicate a role for RecX in the SOS response in mycobacteria.

The complemented strains were applied to a homologous recombination assay, to study the role of RecX on the recombination activity of RecA *in vivo*. It was found for *M. smegmatis* that the frequency of double crossover events was significantly higher for *recA+recX*, at 0.261 compared to  $\Delta recX$  at 0.17. This value represents the ratio of double crossovers: single crossovers+random integrants.

The fully complemented strain was used as the equivalent of the wild type control. It has been reported that for this recombination assay, the fully complemented strain gives

recombination values which are very close to the values for the wild type (Papavinasasundaram, *et al.*, 1998).

Experiments were also carried to study the effect of RecX on RecA levels following a pulse of DNA damage. Differences were found for RecA levels between the *recA+recX* and  $\Delta recX$  strains, showing that RecX plays a role in the recovery of RecA levels following DNA damage. RecX speeds up the gradual reduction of RecA levels. The down-regulation of RecA by RecX as found in this study could lead to an acceleration of the shutdown of the SOS response.

In conclusion, the results of this study suggest that in *M. smegmatis* RecX affects RecA function *in vivo* by increasing the frequency of homologous recombination, increasing cell survival following exposure to DNA damage and influencing the recovery levels of RecA following pulse DNA damage treatment to speed the shutting-down of the SOS response.

## CHAPTER 6

### GENERAL DISCUSSION

#### 6.1 Summary of experiments carried out to identify the mode of interaction between RecX and RecA (and *recA*)

The *M. tuberculosis* RecX protein was purified and used in a DNA gel shift assay to investigate if the RecX protein binds to the upstream promoter region of *recA* to modulate RecA expression by transcriptional control. The result of this assay showed no binding of RecX to the *recA* upstream promoter regions of *M. smegmatis* or *M. tuberculosis*. This suggests no DNA-protein interaction between *recA* and RecX and therefore suggests that RecX does not bind to the *recA* promoter region to influence the expression of *recA*.

A forward yeast-two hybrid assay was carried out to determine if RecX affects RecA activity by means of a protein-protein interaction between the two proteins *in vivo*. The results of this assay suggest that the RecA and RecX interact directly with each other. Positive results were also obtained for the interaction between RecA and RecA, consistent with the fact that RecA forms complexes with itself. Therefore it seems probable that RecX modulates RecA activity by the direct interaction of the two proteins.



## **6.2 Summary of experiments carried out to assess the influence of RecX on RecA**

### **ATPase activity and RecA-stimulated LexA cleavage activity *in vitro***

It was observed from these assays that RecX has an inhibitory effect on both the ATPase and LexA cleavage activities of RecA. An increased concentration of RecX, tested up to a RecA:RecX ratio of 1: 3, results in increased levels of inhibition of LexA cleavage activities of RecA. It was found for the ATPase assays that there is a higher level of inhibition when RecX is added to the reaction before RecA activation. No obvious difference was observed for the LexA cleavage assay. However it is possible that there may be a difference which might be observed if the bands were quantified by volumetric analysis. It was also shown that when the amount of ssDNA was increased to 5x and 10x the original concentration, this did not change the level of inhibition of LexA cleavage caused by RecX, making it unlikely that RecX inhibits RecA activity by sequestering or directly interacting with the ssDNA to make it unavailable to RecA. RecX was included in a pH dependent LexA cleavage assay in which LexA is cleaved independent of RecA. The addition of RecX to this reaction had no effect on LexA cleavage activity. This points to the likelihood that RecX does not interact directly with LexA to cause inhibition.

The results show that RecX acts as a negative modulator of RecA ATPase and LexA cleavage activities. This inhibition is specific and concentration dependent. RecX interferes with the activation stage of RecA to have this effect.

### 6.3 Summary of experiments carried out to assess the consequences *in vivo* of the effect of RecX on RecA biochemical activity

To investigate the role of *recX* on the biochemical activities of RecA, strains of a *M. smegmatis* *recA-recX* deletion mutant, complemented with *M. smegmatis* *recA* and *recA+recX* were used in different assays. The fully complemented strain was used as the equivalent of the wild type control as it has been reported for these strains that the fully complemented strain gives recombination values which are very close to the values for the wild type (Papavinasundaram, *et al.*, 1998).

A viability assay was carried out to determine the effect of RecX on cell survival following DNA damage. The results showed a higher percentage survival for *M. smegmatis* *recA+recX*, at 13% compared to  $\Delta recX$ , at 4.3%.

The same strains were applied to a homologous recombination assay. The ratio of double crossovers per strain to the total number of single crossovers and random integrations for a plasmid which was transformed into each strain. It was found for *M. smegmatis* that the frequency of double crossover events was higher for *recA+recX*, at a value of 0.26 compared to  $\Delta recX$  at 0.17.

Experiments carried out to study the effect of RecX on RecA levels following pulse DNA damage showed differences in RecA levels between the *recA+recX* and  $\Delta recX$  strains, measured by Western blots over a time course up to 72 hours. RecA levels peaked at 24 hours in the presence and absence of *recX*; however in the presence of *recX*, the decline

to original RecA levels was much more rapid. This is consistent with the *in vitro* findings that RecX inhibits RecA-stimulated LexA cleavage.

These results show that in *M. smegmatis* RecX affects RecA function *in vivo* resulting in increased cell survival following exposure to DNA damage, increased frequency of homologous recombination and altered recovery of RecA levels following pulse DNA damage treatment to speed the shutting-down of the SOS response.

#### **6.4 The role of RecX in mycobacteria: common conclusions from this study**

The results obtained for the yeast-two hybrid assay and the ability of purified RecX to alter RecA ATPase activity and RecA-dependent LexA cleavage *in vitro* indicate that, in mycobacteria, the most likely mode of action of RecX is to modulate RecA activity by a protein-protein interaction.

This conclusion is supported by the following observations:

(1) The inhibitory effect of RecX on RecA is specific to RecX, (2) the inhibitory effect of RecX on RecA is concentration dependent, (3) the inhibitory effect of RecX on RecA is not mediated by sequestration of ssDNA, and (4) RecX does not affect RecA-independent LexA cleavage. Furthermore, the inhibition was found to be more pronounced if RecX is added prior to RecA activation, suggesting that RecX interacts directly with RecA to block the formation of the active nucleoprotein filament. This would have the effect of reducing the signal for RecA-stimulated LexA cleavage.

The ability of RecX to inhibit RecA-dependent LexA cleavage *in vivo* as well as *in vitro* was indicated by the influence of RecX on the recovery of RecA levels following DNA damage.

Additional consequences of RecX modulating RecA activity *in vivo* were increased survival following DNA damage and increased frequency of homologous recombination. These phenotypes could arise from a combination of RecX causing altered regulation of some DNA-damage inducible genes and RecX affecting RecA activity directly, perhaps to prevent excessive formation of RecA nucleoprotein filaments. The increase in cell survival following DNA damage in the presence of RecX reflects the previously observed requirement for RecX for cell survival when RecA is over expressed, as the RecA levels would be elevated following DNA damage.

In conclusion, this study was carried out to test the hypothesis that RecX acts by down regulating either the expression of *recA* or the activity of the RecA protein in mycobacteria. The results obtained support the hypothesis that RecX does indeed act as a modulator of RecA by regulating the activity of the RecA protein.

### **6.5 The findings of this study in the context of current knowledge on RecX**

When this research study was started, homologues of *recX* had been identified in various bacterial species and it had been shown that *recX* is essential for survival when *recA* is over expressed in *P. aeruginosa* (Sano, 1993) and *M. smegmatis* (Papavinasasundaram *et al.*, 1998). However the precise role of RecX was largely unknown.

This study has demonstrated that RecX modulates RecA activity *in vitro* and has identified various roles of RecX in mycobacterial cells (see conclusions, above). During the course of this work and following it, research by other groups has also been carried out on RecX in mycobacteria and various other bacterial species. The results of this study have mostly been confirmed by other findings, and these have subsequently been built upon so that we now have a much more detailed understanding of the role of RecX in the cell.

Consistent with the lack of RecX binding to the *recA* promoter region observed here, deletion of *recX* in *E. coli* and *Streptomyces lividans* does not affect *recA* expression (Pages *et al.*, 2003, Vierling *et al.*, 2000). Therefore, transcriptional control of *recA* by RecX is unlikely.

The demonstration by a yeast two-hybrid assay that RecX interacts directly with RecA is also supported by results found for *E. coli*, in which RecA and RecX proteins have also been shown to physically interact through a yeast two-hybrid analysis (Stohl *et al.*, 2003). The yeast two-hybrid assay is known to give false positives however other studies have also demonstrated a physical interaction between mycobacterial RecX and RecA using complementary methods such as affinity chromatography and immunoprecipitation (Venkatesh *et al.*, 2002).

The results showing that RecX interacts with RecA to inhibit ATPase and RecA-stimulated LexA cleavage activities *in vitro* have also been demonstrated in *E. coli* and *Deinococcus radiodurans* (Stohl *et al.*, 2003; Sheng *et al.*, 2005). *In vitro* studies in *M. tuberculosis* have also shown that RecX inhibits ATPase activity of RecA (Venkatesh *et al.*, 2002).

Structural studies and molecular modelling have lead to the proposal that RecX binds within the deep helical groove of the RecA nucleoprotein filament, where LexA binds and strand exchange occurs, (Vanloock *et al.*, 2003; Mishra *et al.*, 2003). RecX is predicted to simultaneously interact with the ATP binding and the DNA binding sites of RecA (Mishra *et al.*, 2003). The RecX molecule sits on the RecA filament at its ATP binding site. This inhibits entry of ATP, thereby reducing ATPase activity. Part of the RecX molecule also sits along the helix axis, therefore preventing the vertical entry of the DNA molecule. This process inhibits ATPase activity and RecA-stimulated LexA cleavage, therefore also inhibiting the SOS response (Mishra *et al.*, 2003). Furthermore, it has also been proposed that RecX is able to inhibit ATPase activity as it binds to three consecutive RecA molecules at the ATP binding site, which is close to the DNA binding site (Vanloock *et al.*, 2003). RecX binds from the C-terminal domain of one subunit to the nucleotide-binding core of another subunit, inactivating RecA's ATPase activity and preventing the C terminus from undergoing the large conformational changes that are associated with different nucleotide-binding states (Vanloock *et al.*, 2003).

An alternative mechanism has also been proposed in which RecX blocks filament extension by a capping mechanism at the end that extension occurs. The end-dependent disassembly of RecA filaments continues during this process, resulting in a net disassembly of RecA filaments and therefore a gradual loss of RecA function (Drees *et al.*, 2004a).

The results from this study showing that the percentage of double crossovers is lower in the absence of RecX in *M. smegmatis* was initially surprising as it has been found in *M. tuberculosis* and *E. coli* that RecX inhibits strand exchange promoted by RecA *in vitro* and it has been suggested that RecX might act as an anti-recombinase to quell inappropriate recombinational repair during normal DNA metabolism (Venkatesh *et al.*, 2002). However, the effect of RecX on homologous recombination seems to vary between species. In *Deinococcus radiodurans* the recombination frequency was higher in the absence of *recX* (Sheng *et al.*, 2005). In *S. lividans* as well as in *E. coli* it has been found that a *recX* mutation does not appear to affect homologous recombination by RecA (Vierling *et al.*, 2000; Lusetti *et al.*, 2006). In contrast the efficiency of the recombination mediated processes of pilus variation and natural transformation was reduced in *Neisseria gonorrhoeae* lacking *recX* (Stohl *et al.*, 2001). It is possible that in some species the inhibition of strand exchange activity mediated by RecX is counteracted by other factors *in vivo*.

The results from this study showing that RecX has an effect on RecA levels following pulse DNA damage, are supported by other studies. It has been shown in *E. coli* that

RecX modulates RecA levels and that over expressing RecX leads to a decrease in RecA levels (Stohl *et al.*, 2003). It has also been shown that RecX levels are elevated during the SOS response in *E. coli* (Pages *et al.*, 2003) and that in *Streptomyces lividans*, *recA* is always expressed at a basal level with the *recA-recX* transcript only being expressed upon DNA damage (Vierling *et al.*, 2000).

The values obtained for the survival experiments show a difference between the two strains unlike the results obtained by Papavinasasundaram, *et al.*, (1998) for experiments to study the role of *recX* in survival following DNA damage by UV, in which no difference was seen. However these two experiments differed in the types of DNA damage being induced and also the times of exposure.

More recent research has shown that RecX is a part of a network of proteins which act to regulate RecA activity, of which DinI and RecF are of particular significance in relation to RecX (Lusetti *et al.*, 2004, 2006; Renzette *et al.*, 2007). Much of the regulation is focused on RecA filament assembly and disassembly (Baitin *et al.*, 2008).

DinI has an antagonistic effect to RecX on RecA regulation (Lusetti *et al.*, 2004; Renzette *et al.*, 2007). It has been found that unlike the effect of RecX on RecA, the DinI protein modulates and stabilises RecA filaments during the SOS response (Lusetti *et al.*, 2004).



DinI expression is induced by DNA damaging agents and it acts to stabilise RecA filaments by binding to RecA protein monomers at the disassembly end of the filament. DinI is able to do this by replacing the RecX that is bound at the end where RecA filament extension is occurring. In this way, DinI is able to suppress the end-dependent disassembly action of RecX while extension of the RecA filament occurs (Lusetti *et al.*, 2004).

RecF sequesters RecX through direct interaction and so RecX is unable to inhibit filament extension. RecF thus facilitates RecA protein filament extension by antagonizing RecX function (Lusetti *et al.*, 2006).

Recent studies have also shown that SSB antagonizes a RecX-RecA interaction and the mechanism of RecX action may involve RecX binding to both RecA protein and to DNA. It has been suggested that the interaction of RecX protein and RecA may enable an enhanced DNA binding by RecX protein (Baitin *et al.*, 2008).

On going studies on RecX and its regulation of RecA activity, homologous recombination, and its role in DNA repair in general have continued to arouse interest and to reveal new facets to add to our understanding of the role of RecX.

## 6.6 Future Work

There are various reasons for the continued interest in the study of RecX, particularly as a modulator of RecA function.

As described in the Introduction, RecA is a key protein in pathways leading to the repair of DNA following DNA damage. Such DNA damage is caused by the exposure of mycobacteria to ROIs and RNIs while these organisms reside in the macrophage.

Therefore understanding the mechanisms by which RecA functions and is regulated is significant in our understanding of the basic biology of how mycobacteria are able to evade the host immune response. In addition, many antibiotics lead to bacterial DNA damage which is repaired by RecA (Wigle and Singleton, 2007). Therefore developing RecA inhibitors could enhance the toxicity of antibiotics. In addition RecA is involved in the mechanisms which lead to the development of antibiotic resistance (Wigle and Singleton, 2007). Therefore RecA inhibitors could be used to delay or prevent the appearance of this. This is particularly significant in the treatment of tuberculosis which involves a long regimen of antibiotic treatments and the treatment of which has led to the development of MDR and XDR strains of *M. tuberculosis*. Antibiotic resistance modifying agents could be given in conjunction with antibiotics to prevent or delay mutations from occurring which lead to the development of bacterial antibiotic resistance. This would also be relevant to other organisms aside from *M. tuberculosis*. Indeed, studies have been carried out to screen for RecA ATPase inhibitors for the suppression of the evolution of antibiotic resistance by the inhibition of *E. coli* RecA (Lee *et al.*, 2005; Wigle and Singleton, 2007).

This study has shown RecX to be a modulator of RecA function and these findings have been confirmed by subsequent studies. This is a relevant contribution to our understanding of the basic biology of DNA repair in mycobacteria and indeed other prokaryotes. The mechanism of action of RecX has been further defined in detail and a network of regulatory proteins has been uncovered through subsequent work.

Further work on RecX and its structural biology and mechanisms of action continues. It could be interesting to explore the possibility of using RecX as a potential drug target, as it is now known that RecX blocks the activation of RecA by capping filament extension and therefore also all RecA functions. It has been shown that cell survival following DNA damage and homologous recombination are significantly reduced in the absence of RecX.

More directly related to the work carried out in this study, future work could involve looking at the cell survival and recombination assays in *M. tuberculosis* itself, using  $\Delta recX$  and fully complemented strains.

It would also be interesting to study the effects of RecX on RecA-dependent LexA cleavage and ATPase activities *in vivo*. For example with an *in vivo* LexA cleavage assay, it may be possible to monitor expression of a gene solely regulated by LexA by a more quantitative method.

Various experiments were carried in this study which gave no conclusive results. These include a time course experiment to study the effect of RecX on LexA levels following pulse DNA damage, a study to look at the effect of RecX on LexA cleavage following DNA damage assessed by gel shift, an *in vivo* LexA cleavage assay, a RecA and RecX DNA binding assay assessed by gel shift. Although these did not give conclusive results, it would be interesting to pursue them further, perhaps with some modifications.

It would be interesting to explore how the network of proteins involved in RecA regulation in *E. coli* function in mycobacteria. It is possible that a different or varied regulatory system exists in mycobacteria. This system may even vary between slow and fast growing mycobacteria and differences may be relevant to how RecA function and DNA repair occur in pathogenic species. It is also possible that RecX is involved in other functions aside from RecA regulation.

Ongoing studies in other organisms on the mechanism of RecX function may also in time reveal other proteins which regulate RecA function, adding to the network of proteins we now know about and more detailed understanding of the mechanisms of DNA repair in the cell.

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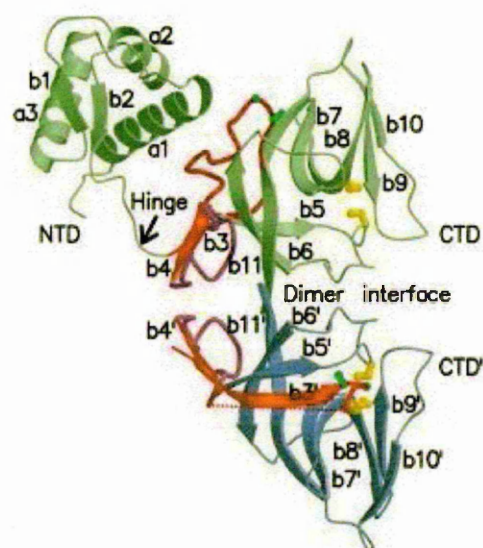
Nucleotide excision repair- and polymerase eta-mediated error-prone removal of mitomycin C interstrand cross-links. *Mol Cell Biol* **23**(2):754-61.

Website for ANOVA analysis:

<http://faculty.vassar.edu/lowry/VassarStats.html>

## **APPENDIX**

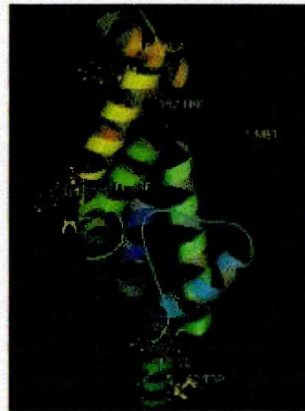
**FIGURE 29: Structure of the LexA protein dimer**



The structure of the LexA protein dimer from *E. coli*  
The structure includes the N and C terminal domains  
and the dimer interface.  
Taken from Luo *et al.*, 2001



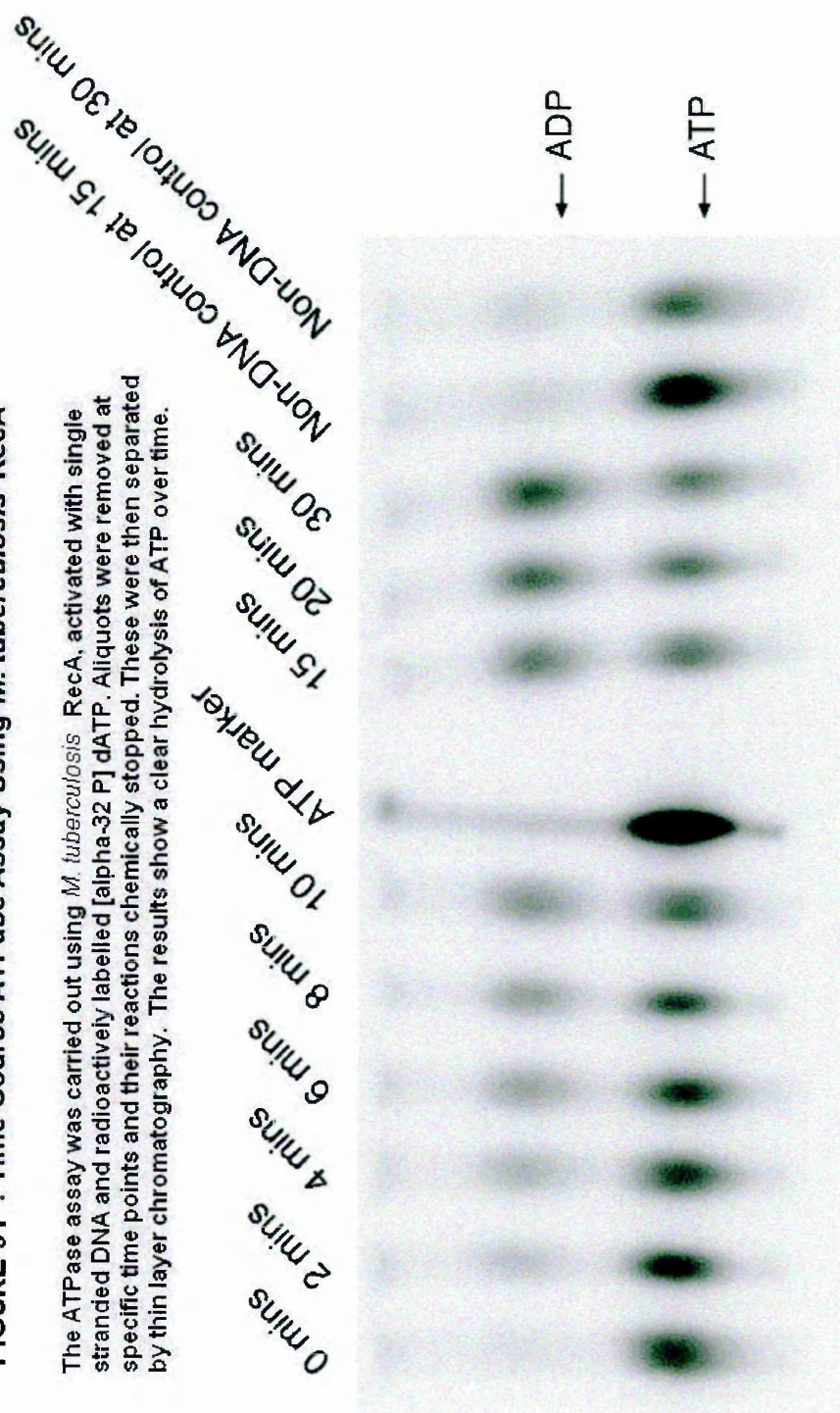
**FIGURE 30: Structure of the RecX protein monomer**



Structure of the RecX protein monomer of *E. coli* based on molecular modelling, showing the main protein residues.  
Taken from Mishra *et al.*, 2003

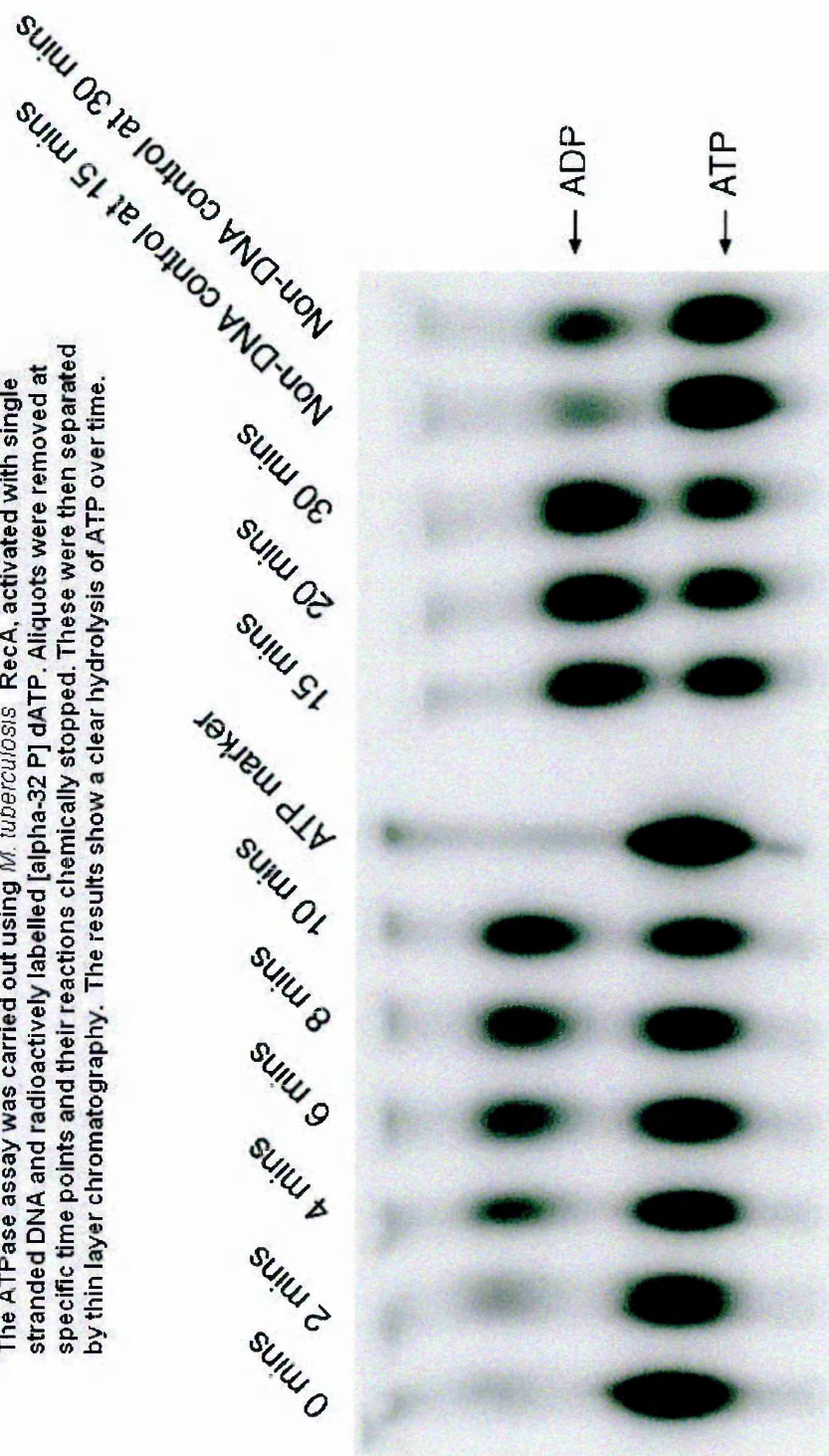
**FIGURE 31 : Time Course ATPase Assay Using *M. tuberculosis* RecA**

The ATPase assay was carried out using *M. tuberculosis* RecA, activated with single stranded DNA and radioactively labelled [alpha-32 P] dATP. Aliquots were removed at specific time points and their reactions chemically stopped. These were then separated by thin layer chromatography. The results show a clear hydrolysis of ATP over time.



**FIGURE 32: Time Course ATPase Assay Using *M. tuberculosis* RecA**

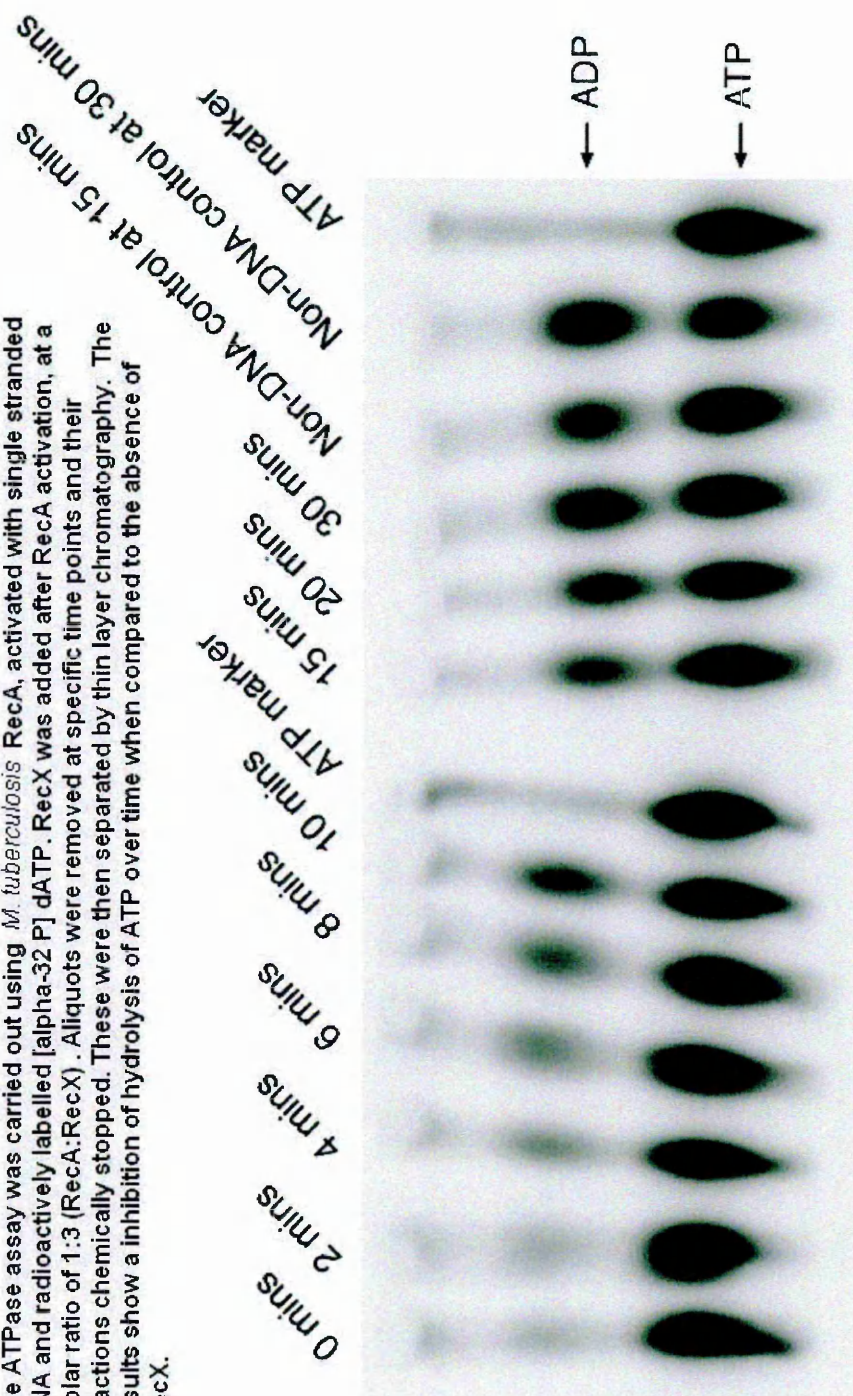
The ATPase assay was carried out using *M. tuberculosis* RecA, activated with single stranded DNA and radioactively labelled [ $\alpha$ - $^{32}$ P] dATP. Aliquots were removed at specific time points and their reactions chemically stopped. These were then separated by thin layer chromatography. The results show a clear hydrolysis of ATP over time.





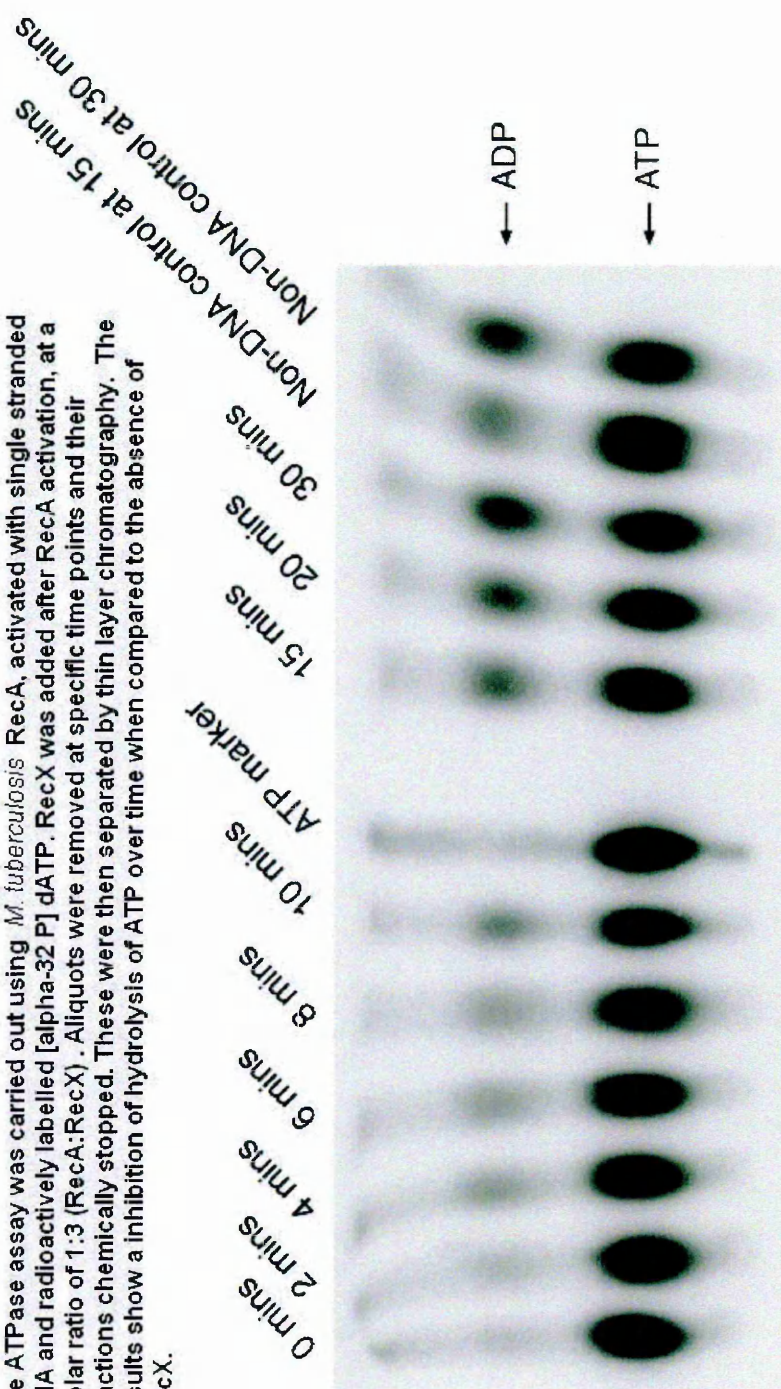
**FIGURE 33: Time Course ATPase Assay Using *M. tuberculosis* RecA and RecX**

The ATPase assay was carried out using *M. tuberculosis* RecA, activated with single stranded DNA and radioactively labelled [alpha-32 P] dATP. RecX was added after RecA activation, at a molar ratio of 1:3 (RecA:RecX). Aliquots were removed at specific time points and their reactions chemically stopped. These were then separated by thin layer chromatography. The results show a inhibition of hydrolysis of ATP over time when compared to the absence of RecX.



**FIGURE 34: Time Course ATPase Assay Using *M. tuberculosis* RecA and RecX**

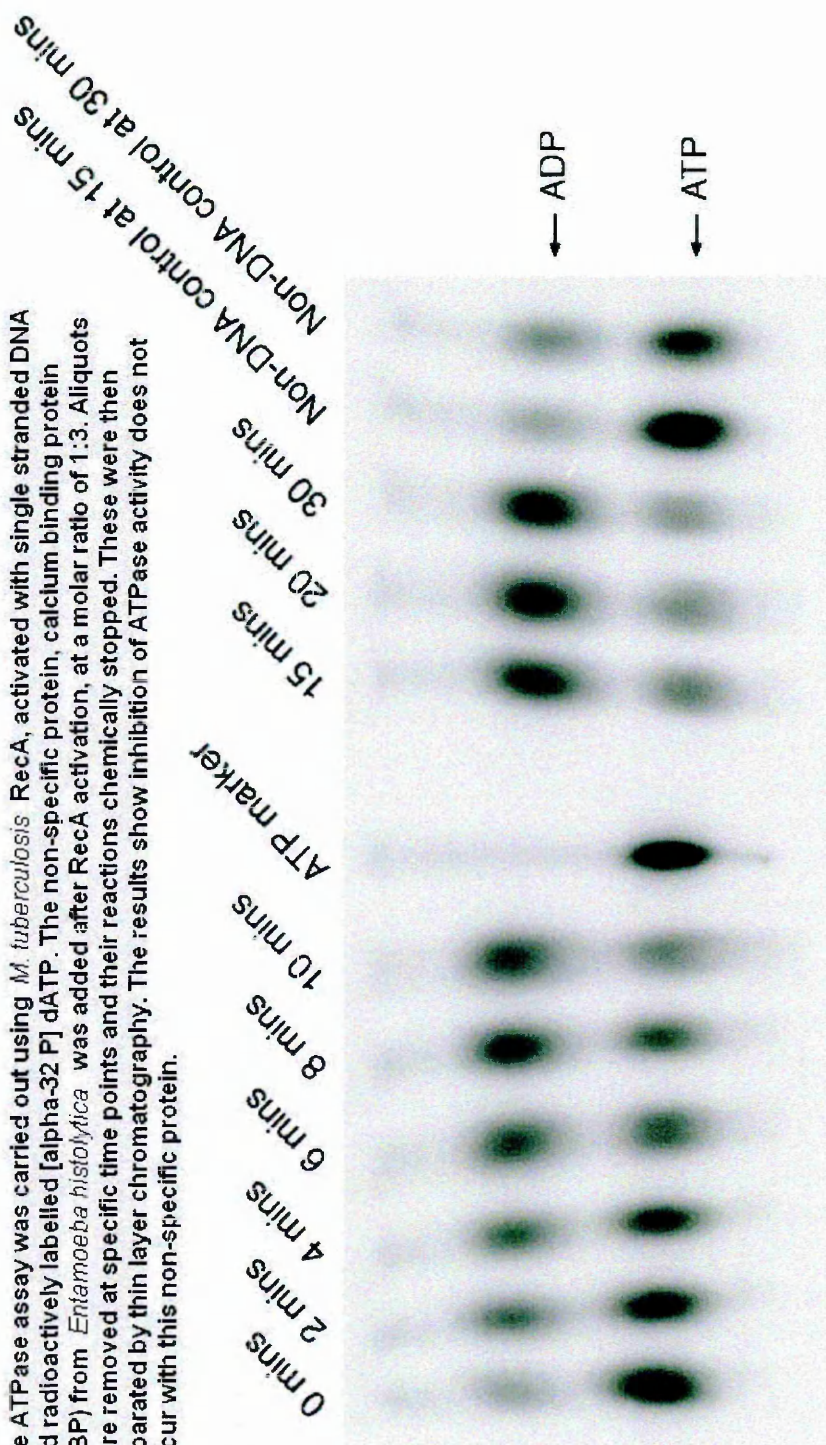
The ATPase assay was carried out using *M. tuberculosis* RecA, activated with single stranded DNA and radioactively labelled [ $\alpha$ - $^{32}$ P] dATP. RecX was added after RecA activation, at a molar ratio of 1:3 (RecA:RecX). Aliquots were removed at specific time points and their reactions chemically stopped. These were then separated by thin layer chromatography. The results show a inhibition of hydrolysis of ATP over time when compared to the absence of RecX.





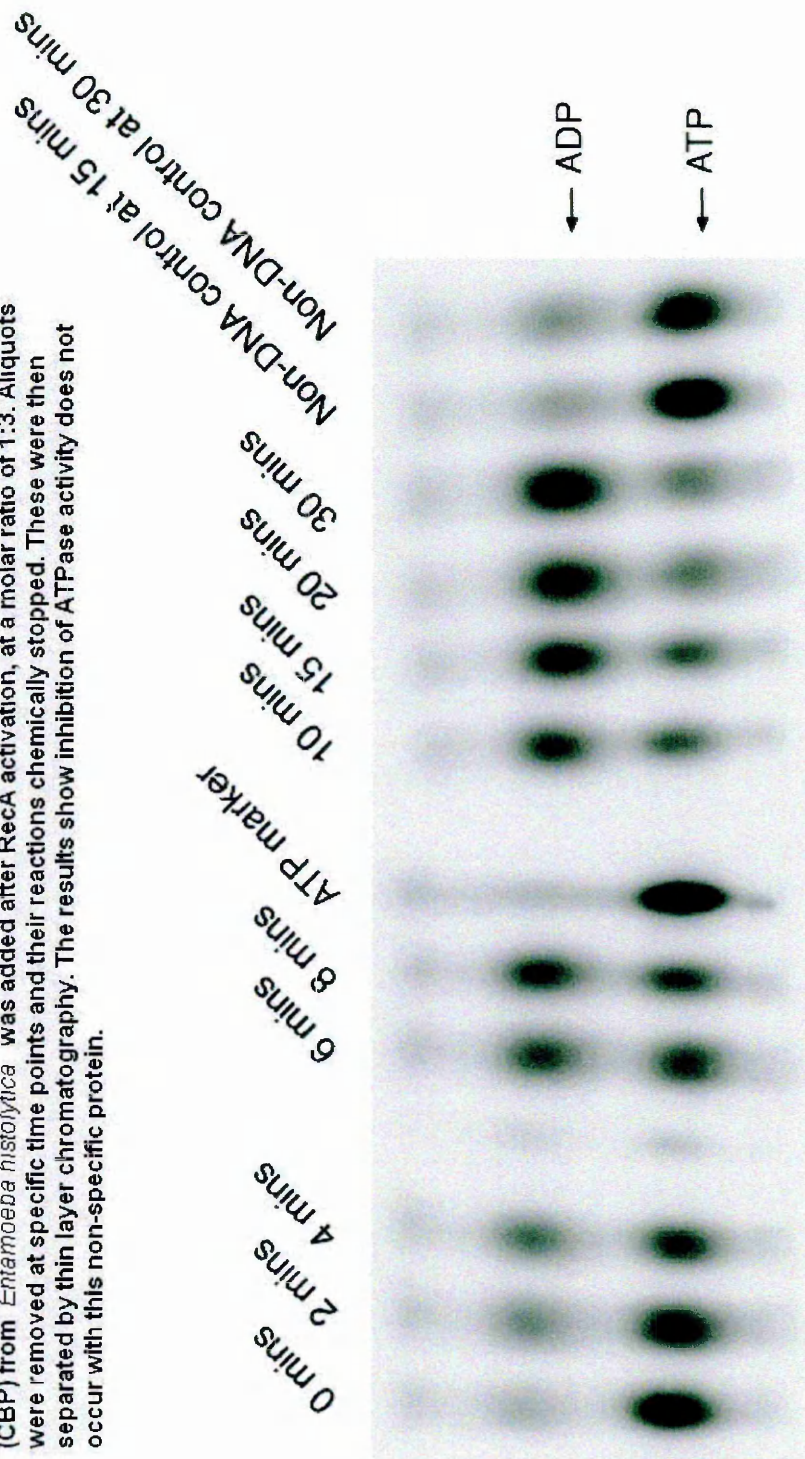
**FIGURE 35: Time Course ATPase Assay Using *M. tuberculosis* RecA and Calcium Binding Protein**

The ATPase assay was carried out using *M. tuberculosis* RecA, activated with single stranded DNA and radioactively labelled [ $\alpha$ - $^{32}$ P] dATP. The non-specific protein, calcium binding protein (CBP) from *Entamoeba histolytica* was added after RecA activation, at a molar ratio of 1:3. Aliquots were removed at specific time points and their reactions chemically stopped. These were then separated by thin layer chromatography. The results show inhibition of ATPase activity does not occur with this non-specific protein.



**FIGURE 36: Time Course ATPase Assay Using *M. tuberculosis* RecA and Calcium Binding Protein**

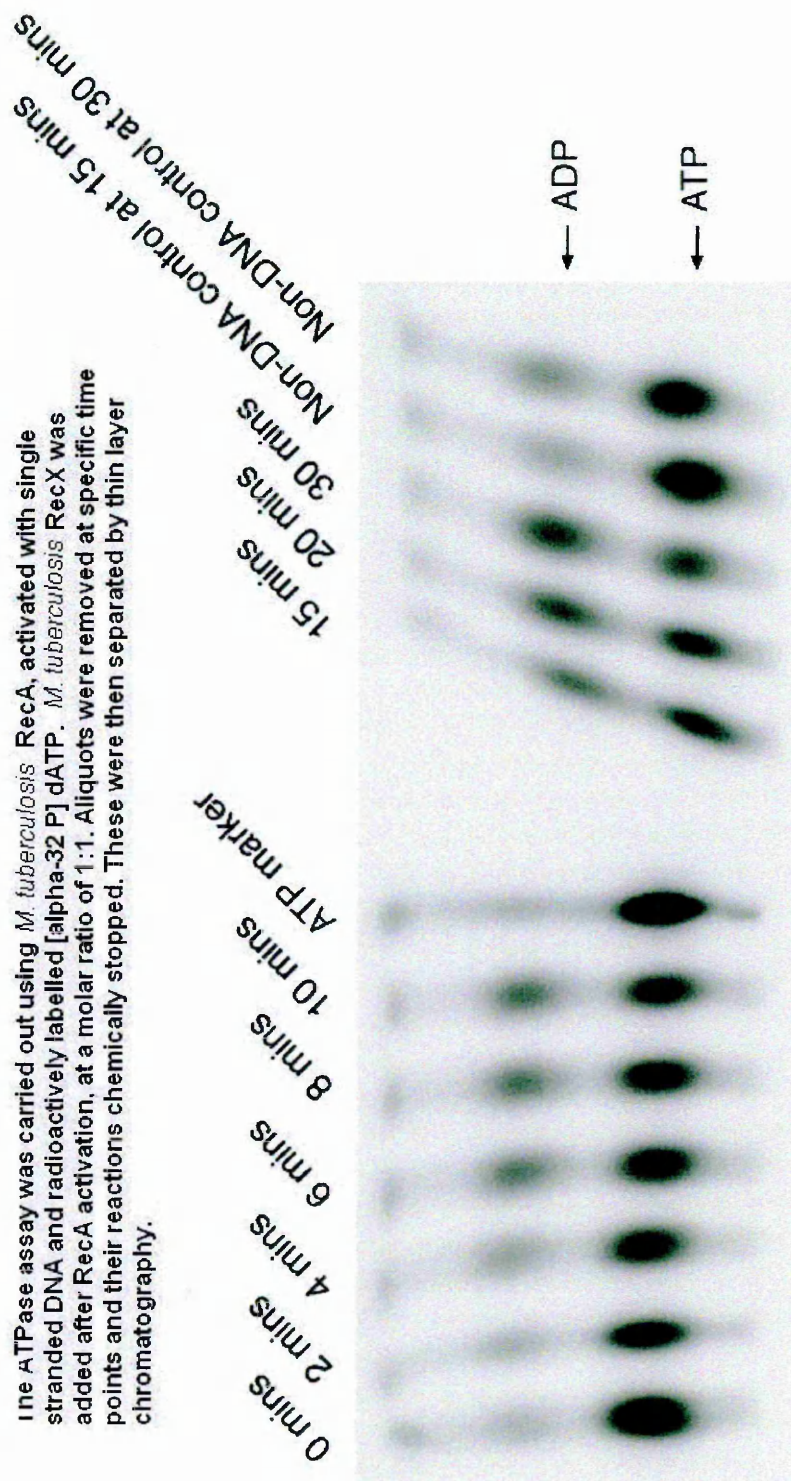
The ATPase assay was carried out using *M. tuberculosis* RecA, activated with single stranded DNA and radioactively labelled [ $\alpha$ - $^{32}$ P] dATP. The non-specific protein, calcium binding protein (CBP) from *Entamoeba histolytica* was added after RecA activation, at a molar ratio of 1:3. Aliquots were removed at specific time points and their reactions chemically stopped. These were then separated by thin layer chromatography. The results show inhibition of ATPase activity does not occur with this non-specific protein.





**FIGURE 37: Time Course ATPase Assay Using *M.tuberculosis* RecA and RecX in a 1:1 Ratio**

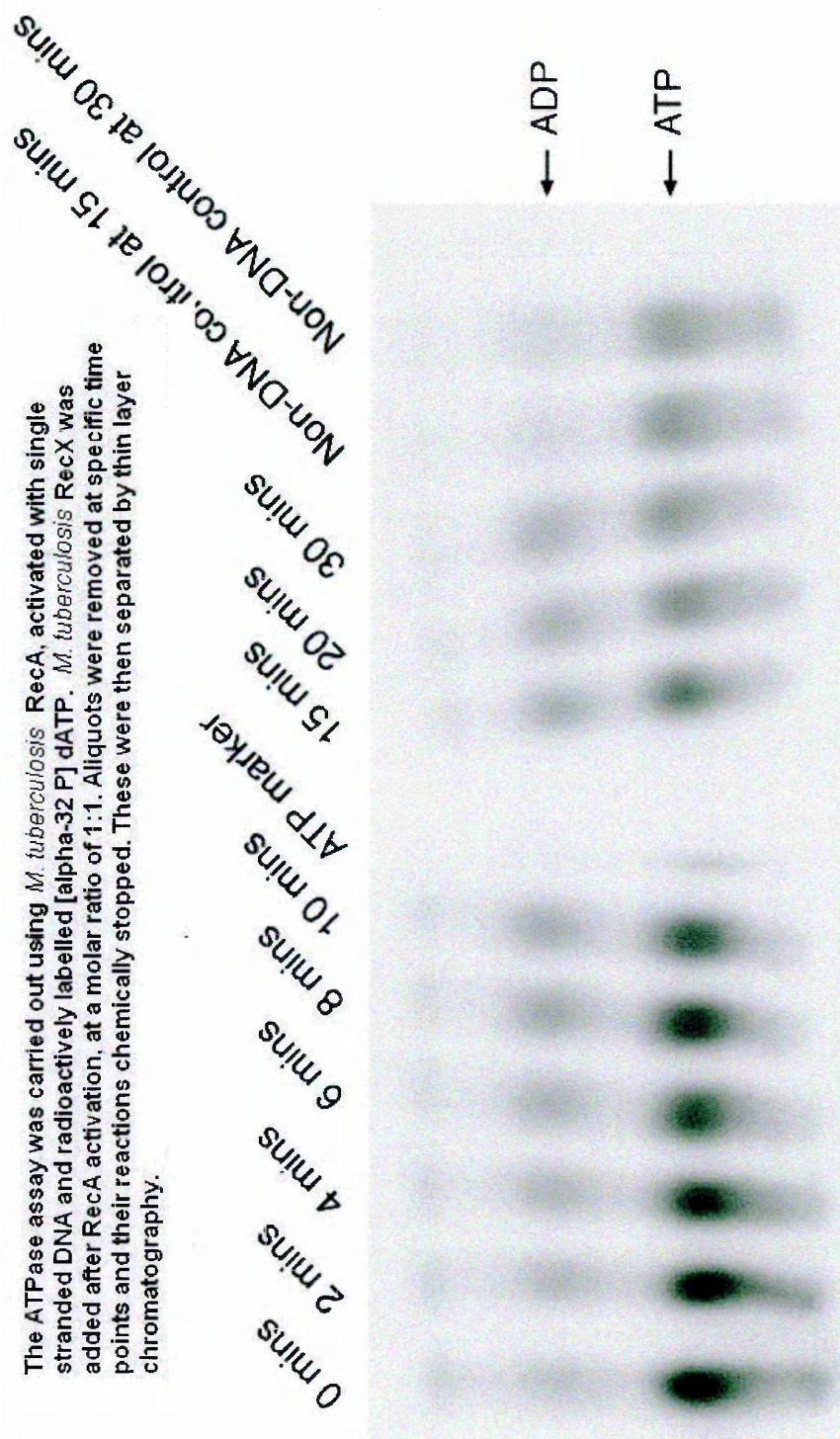
The ATPase assay was carried out using *M. tuberculosis* RecA, activated with single stranded DNA and radioactively labelled [ $\alpha$ - $^{32}$ P] dATP. *M. tuberculosis* RecX was added after RecA activation, at a molar ratio of 1:1. Aliquots were removed at specific time points and their reactions chemically stopped. These were then separated by thin layer chromatography.





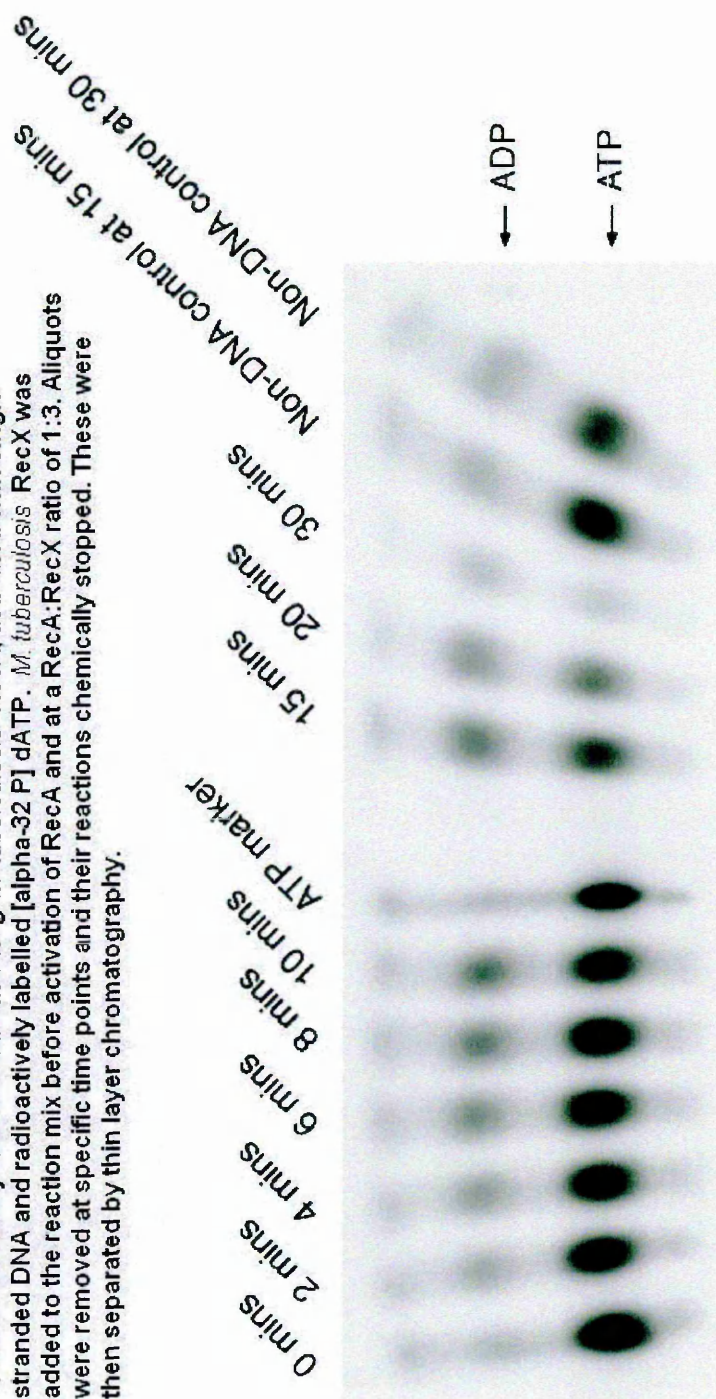
**FIGURE 38: Time Course ATPase Assay Using *M.tuberculosis* RecA and RecX in a 1:1 Ratio**

The ATPase assay was carried out using *M. tuberculosis* RecA, activated with single stranded DNA and radioactively labelled [ $\alpha$ - $^{32}$ P] dATP. *M. tuberculosis* RecX was added after RecA activation, at a molar ratio of 1:1. Aliquots were removed at specific time points and their reactions chemically stopped. These were then separated by thin layer chromatography.

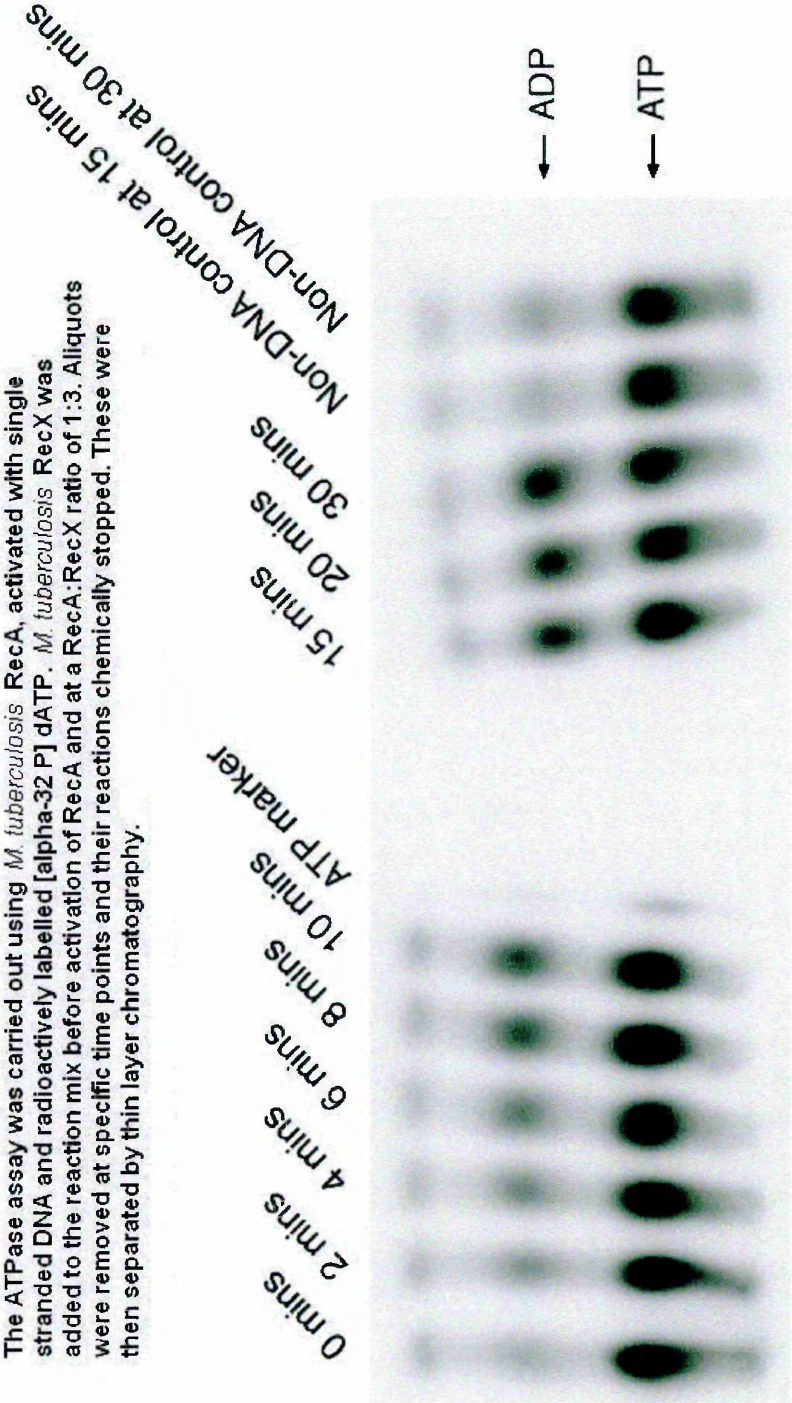


**FIGURE 39: Time Course ATPase Assay Using *M. tuberculosis* RecA and Adding RecX Before RecA Activation**

The ATPase assay was carried out using *M. tuberculosis* RecA, activated with single stranded DNA and radioactively labelled [ $\alpha$ - $^{32}$ P] dATP. *M. tuberculosis* RecX was added to the reaction mix before activation of RecA and at a RecA:RecX ratio of 1:3. Aliquots were removed at specific time points and their reactions chemically stopped. These were then separated by thin layer chromatography.

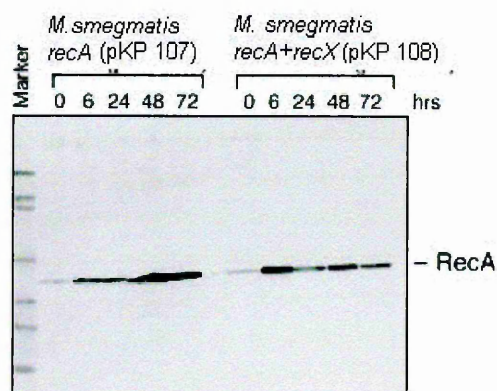


**FIGURE 40: Time Course ATPase Assay Using *M. tuberculosis* RecA and Adding RecX Before RecA Activation**



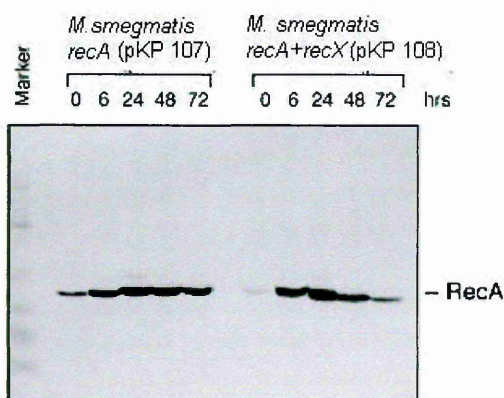


**FIGURE 41: Western analysis showing variation in RecA levels following pulse DNA damage for *M. smegmatis* *recA-recX* deletion strains complemented with *recA* or *recA+recX***



A *M. smegmatis* *recA-recX* deletion mutant complemented with *M. smegmatis* *recA* (pKP107) and *recA+recX* (pKP108) constructs was treated with a pulse of mitomycin C for one hour. The cultures were then washed with Dubos medium and incubated in medium without mitomycin C for a period of 72 hours. Regular harvests were prepared at 0, 6, 24, 48 and 72 hours. The cell free extracts were prepared and RecA levels were analysed by western blots using RecA antibodies. The experiment was repeated 3 times and the figure shows a typical blot.

**FIGURE 42: Western analysis showing variation in RecA levels following pulse DNA damage for *M. smegmatis* *recA-recX* deletion strains complemented with *recA* or *recA+recX***



A *M. smegmatis* *recA-recX* deletion mutant complemented with *M. smegmatis* *recA* (pKP107) and *recA+recX* (pKP108) constructs was treated with a pulse of mitomycin C for one hour. The cultures were then washed with Dubos medium and incubated in medium without mitomycin C for a period of 72 hours. Regular harvests were prepared at 0, 6, 24, 48 and 72 hours. The cell free extracts were prepared and RecA levels were analysed by western blots using RecA antibodies. The experiment was repeated 3 times and the figure shows a typical blot.